SYMPATHETIC NERVE RESPONSE AND VASCULAR FUNCTION:

EFFECTS OF INFLAMMATION ON NERVE-MEDIATED VASOCONSTRICTION IN THE RAT TAIL ARTERY

By

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Abstract

Chronic inflammation contributes to changes in vascular function in a range of vascular diseases. Whether these vascular changes in function are nerve-mediated remains unknown. Sympathetic nerve-mediated responses in isolated tail arteries of rats in an experimentally induced chronic inflammatory state were examined. Electrical field stimulations between 1.25 and 40 Hz resulted in frequency-dependent contractions sensitive to tetrodotoxin. Neurogenic contractions in blood vessels of experimental animals were significantly greater compared to those of control animals. Alpha$_1$-adrenoceptor antagonists significantly inhibited contractile responses at lower frequencies of stimulation (1.25 – 5 Hz) in blood vessels of experimental rats compared to controls. Alpha$_2$-adrenoceptor antagonism had no effect on electric field-evoked vasoconstrictions in experimental and control animals. Inhibition of neuronal reuptake by cocaine comparably enhanced field-stimulated responses in blood vessels of experimental and control animals. Furthermore, the expression of α_1- and α_2-adrenoceptors was different in the endothelium of blood vessels of treated animals compared to control animals. The observation of enhanced neurogenic contraction in blood vessels of animals in a chronic state of inflammation may be attributed to alterations in responsiveness and/or distribution of post-junctional α_1-adrenoceptors.

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List of Abbreviations and Symbols

ATP: Adenosine triphosphate

AC: Adenylyl cyclase

AR: Adrenoceptor

α: Alpha adrenoceptors

ANS: Autonomic nervous system

β: Beta adrenoceptors

CNS: Central Nervous System

Co: Cocaine; sodium channel blocker

CFA: Complete Freund's Adjuvant

cAMP: Cyclic adenosine monophosphate

DAG: Diacylglycerol

EFS: Electrical field stimulation

GPCR: Guanine Protein-coupled receptors

HUVEC: Human umbilical vein endothelial cells

HPA: Hypothalamic-Pituitary-adrenal Axis

IHC: Immunohistochemistry

IP_3: Inositol 1,4,5-triphosphate

ICAM: Intercellular adhesion molecules

IFNγ: Interferon factor neuron gamma

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-10: Interleukin 10

NPY: Neuropeptide Y

NA: Noradrenaline

NFκβ: Nuclear factor kappa β

PAMP: Particle-associated molecular pattern

PLC: Phospholipase C

Pra: Prazosin; α_1 receptor antagonist

PKC: Protein Kinase C

Rau: Rauwolscine; α_2 receptor antagonist

SBP: Systolic blood pressure

SNS: Sympathetic nervous system

TTX: Tetrodotoxin

TLR: Toll-like receptors

TNF- α: Tumor necrosis factor- α

VCAM: Vascular cell adhesion molecules

VSMC: Vascular smooth muscle cell

WB: WB4101; α_1 and α_2 receptor antagonist

1. Introduction

1.1. The circulatory system

The circulatory system is a “collection of organs” which permits the circulation of blood and the transport of oxygen and nutrients to body organs, and the removal of carbon dioxide and metabolic wastes away from tissues. The transport modality by this system contributes to homeostasis within the body through the constant exchange of nutrients and metabolic materials (Sherwood et al., 2013). The mammalian circulatory system itself consists of the heart as the pump, blood as the transport medium and the blood vessels as the passageway for circulating fluid (i.e. blood); which circulates in a closed circuit between the systemic and pulmonary circulations. This network originates from the heart travelling through the vasculature, arteries, arterioles, capillaries, venules and veins, and back to the heart through both the pulmonary and systemic vasculatures (Sherwood et al., 2013).

1.1.1. The vasculature

The vascular (blood vessel) component of the circulatory system provides the network and the route for the transport of needed material and the removal of waste from cells and tissues. It consists of arteries and veins which make up the macrocirculation, and the arterioles, capillaries and venules which constitute the microcirculation. These blood vessels differ in their constitutive wall layers, comprising endothelial cells, smooth muscle cells and connective tissue. The difference in constitutive wall tissue reflects the function of the individual layer. The innermost layer known as the *tunica intima* consists of a one-cell thick layer of endothelial cells (the endothelium) and the subendothelial space. The middle layer called the *tunica media*, is

the thickest layer and is composed of smooth muscle cells. Finally, the outermost layer, the *tunica adventitia* is composed mainly of fibrous and elastic connective tissue as well as sympathetic nerve endings (Figure 1). Each of these layers serves a distinct physiological function depending on the type of blood vessel (Sherwood et al., 2013).

1.1.2. Vascular physiology

The innermost cell layer of endothelial cells is the major exchange site for nutrients. This function is possible due to the single cell nature of this layer allowing for semi-permeability and increased surface area at the capillary level (Pries and Kuebler, 2006). For this reason, the capillaries which constitute only the endothelium are the major site of nutrient and waste exchange for the tissues (Pries and Kuebler, 2006; Sherwood et al., 2013). The smooth muscle cell layer, adjacent to the endothelium, allows non-capillary and venule blood vessels to constrict or dilate by contracting and relaxing the smooth muscle, respectively. Vascular smooth muscle cells have specialized receptors (e.g. adrenoceptors) which play an important role in the sympathetic nerve function in blood vessels. Contraction of the muscle layer by sympathetic nerves alters the radius (and lumen) of the vessel and the corresponding flow of blood. This contractile capability of the smooth muscle layer and its ability to vary vessel lumen constitutes the regulation of vascular tone and resistance to flow (Perez, 2005; Zacharia et al., 2013; Tank and Lee Wong, 2015). The smooth cell layer varies in thickness depending on the blood vessel. For example, arteries have a thicker smooth muscle layer compared to capillaries and veins. This permits arteries such as the abdominal aorta to sustain high pressures from the pumping heart and at the same time regulate blood pressure; the reason arteries are considered pressure vessels (Sherwood et al., 2013). Finally, the outermost connective tissue

also varies in elastic and fibrous tissue and nerve tissues, depending on the type of blood vessel. For example, main arteries are highly elastic, allowing them to distend under high pressures, whereas arterioles are highly innervated (with little or no elastic tissues) allowing for direct regulation of resistance through smooth muscle-induced changes in lumen radius. For this reason, arterioles are considered resistance vessels (Sherwood et al., 2013).

Generally, modifications of the structure, morphology, and/or function of vascular tissues have been associated with altered blood vessel responses in pathophysiological states. In clinical settings, vascular diseases caused by conditions such as atherosclerosis are initiated by endothelial cell dysfunction and may be precipitated by the inflammatory response (Togan et al., 2015; Park and Park, 2015; Ross and Glomset, 1973).

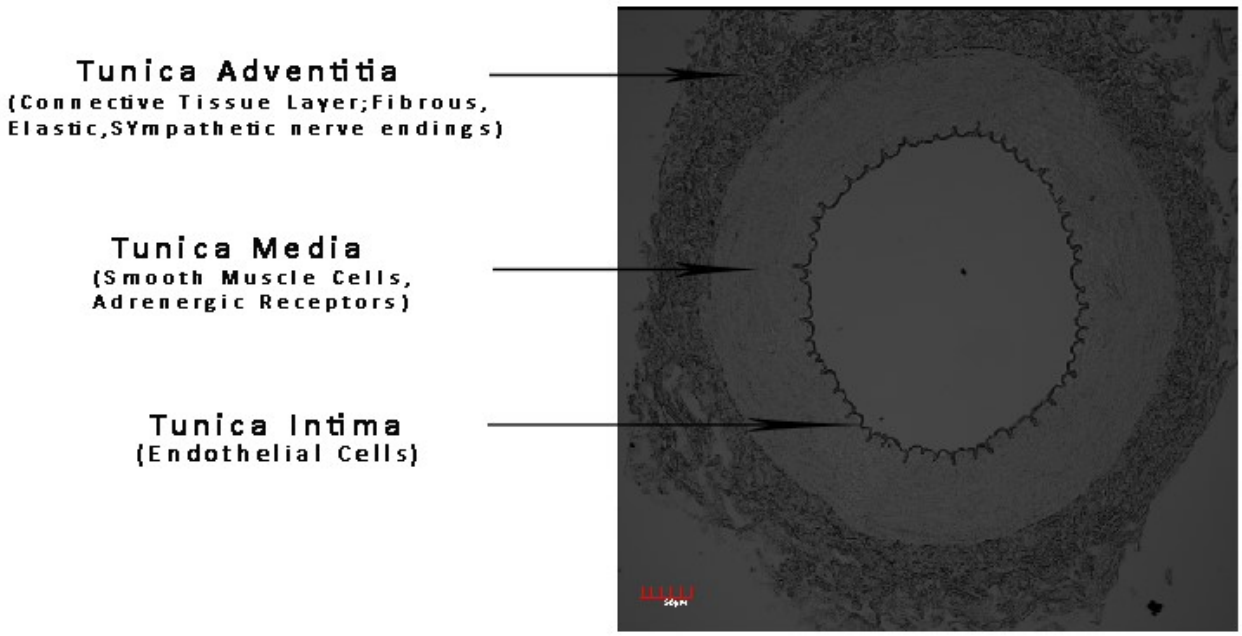


Fig 1. Light micrograph of the cross-section of a tail artery from a saline-treated rat. Micrograph shows arteries consisting of three morphologically distinct layers: tunica adventitia, media and intima. The thicker smooth muscle layer houses the contractile machinery of the vasculature. Image taken under transmitted light at 20X magnification.

1.2. Sympathetic nervous system and nerve function

The sympathetic nervous system (SNS) is broadly defined as a branch of the autonomic nervous system (ANS), responsible for regulating unconscious physiological responses in the body. It acts complementary to the parasympathetic branch to maintain homeostasis. Early studies revealed that two kinds of neurons, the pre- and postganglionic neurons, are involved in neuronal transmission in the SNS. The shorter pre-ganglionic neurons originating from T1-L2 levels of the spinal cord synapse at the sympathetic ganglia, whereas the longer post-ganglionic neurons carry signals from the ganglion to the effector tissues or organs in the entire body (Drake et al., 2005). Stimulation of the sympathetic chain transmits signals via the action of neurotransmitters released from nerve endings. Pre-ganglionic neurons release acetylcholine (ACh) which activates the acetylcholine (nicotinic) receptors present on the post-ganglionic neurons. In response to ACh activation, post-ganglionic neurons release norepinephrine, which interact predominantly with adrenoceptors of the target tissues in the peripheral systems (Tank and Lee Wong, 2015).

Decades of experimental work have provided evidence of the sympathetic nervous system's central role in the regulation of multiple homeostatic mechanisms. Sympathetic nerve fibers supply the internal organs, including the heart, blood vessels, lungs, intestines, genitals, kidneys, salivary and digestive glands and pupils (Drake et al., 2005). Stimulation of the sympathetic nerves has been associated with initiating the "fight and flight" response. In the heart, sympathetic outflow of norepinephrine accelerates the heart rate. In other systems, stimulation of the sympathetic nerves causes constriction of associated blood vessels, relaxation of bronchioles, dilation of pupils, increased sodium and water reabsorption in the

kidneys, reduced peristaltic movement in the gut and inhibition of digestive and salivary gland secretions. Hence, the sympathetic nervous system is able to effect physiological changes through the activation of specialized receptors in target organs.

1.2.1. Adrenoceptors

Adrenoceptors form a class of membrane G protein-coupled receptors (GPCR). Adrenoceptors mediate the physiological effects of the catecholamines norepinephrine and epinephrine in the central and peripheral systems in the body. Norepinephrine and epinephrine are both important in the homeostasis of various systems within the body. Hence, the adrenoceptors to which they bind are widely distributed throughout the body to promote their regulatory functions. Stimulation of the sympathetic nervous system and neurotransmitter binding to these receptors activates the receptor complex causing physiological changes. Activation of the adrenoceptors by neurotransmitter released from the sympathetic nervous system results in various responses depending on the systems involved. In the brain for example, activation of adrenoceptors has been involved in learning and memory (Shakhawat et al., 2012), bronchodilation in the lungs (Paolillo et al., 2013), increased heart rate in cardiac tissue (Capote et al., 2015), regulation of tubular sodium reabsorption in the kidneys via renin release (Mansley et al., 2014), and vascular tone (Wegener et al., 2014; Zacharia et al., 2013). In the cardiovascular system in particular, adrenoceptors mediate constriction and dilation of the blood vessels via contraction and relaxation of the smooth muscle cells. Diameter changes in the vasculature affecting the total peripheral resistance are very important in the regulation of organ blood supply (perfusion) and contribute to the maintenance of vascular tone. This regulation also plays an important role in the metabolic regulation of blood flow to organs to

meet metabolic demands (e.g. during physical exercise) and autoregulatory mechanisms in the brain to ensure constant blood supply at all times (Sherwood et al., 2013).

1.2.2. Classification of adrenoceptor subtypes

In the adrenoceptor family, the two main receptor groups are the alpha (α) adrenoceptors and the beta (β) adrenoceptors. All the same, 20th century ligand binding studies elucidated the structure-function characteristics of the receptors which allowed investigators to classify them in subtypes based on their interactions with agonists and antagonists. Under the alpha receptor category, researchers have classified two sub-categories which are the alpha$_1$ and alpha$_2$ adrenoceptors. There is also evidence of multiple receptor subtypes of the main adrenoceptor types. For example, within the alpha$_1$ type, alpha-1A, -1B and -1D subtypes have been identified. Similarly, alpha-2A, -2B and -2C subtypes have also been identified under the alpha$_2$-adrenoceptor type. In contrast, subtypes of the beta-adrenoceptor type include beta-1, -2 and -3 (Figure 2).

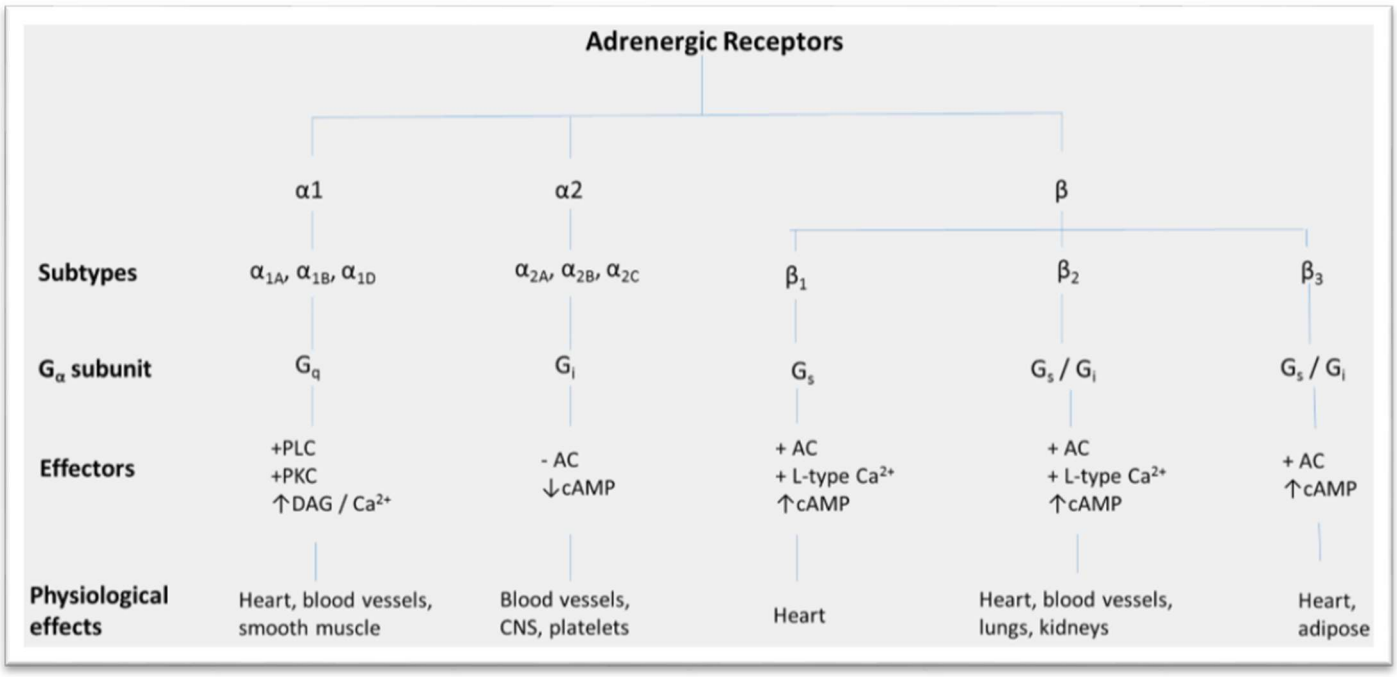


Fig 2. Classification of adrenoceptors, their G-protein and effectors (and physiological effects). PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; AC, adenylyl cyclase; cAMP, cyclic adenosine-3', 5'-monophosphate; +, activation; -, inhibition; ↑, increase; ↓, decrease. (Adapted from Garland and Angus, 1996).

1.2.3. Structure of adrenoceptors

Adrenoceptors, being GPCR's, are integral membrane-spanning proteins that consist of seven transmembrane (TM) domains (Yarden et al., 1986; Pierce et al., 2002). These transmembrane bundles form pockets stabilized by amino acid residues on the helices (figure 3). Furthermore, the structure of the receptors is very important in determining ligand-binding and signal transduction pathways, regulated by the adrenoceptors (Perez, 2006).

Molecular modelling and mutagenesis studies have provided experimental evidence of four structural components involved in the receptor-ligand interactions (Finch et al., 2005). Basically, these experiments conclude that binding of the catecholamine functional groups with the receptor transmembrane (TM) functional pockets form the basis of agonist and antagonist stereo-selectivity of the ligands. Interactions of the catecholamine amino group and the helix carboxylate group create ionic interactions between the ligand and the helix residues of the receptors (Finch et al., 2005; Bockaert et al., 2003). Secondly, interaction between the catechol meta- and para-hydroxyl groups with the serine residues of the TM helix create hydrogen bonding between the agonist and the receptor putative transmembrane helix. Interaction between the phenyl group and the aromatic groups of the helix residues constitute aromatic-aromatic interactions for agonist binding. These interactions are all essential for ligand-receptor binding and define the affinity of an agonist for the receptor. Studies on receptor gene mutations have also demonstrated that changes in TM helix or ligand residue affect the receptor-ligand interaction and hence the activation of the receptor (Strader et al., 1987). Mutations of serine molecules from the particular TM domains affect the receptor subtype

selectivity and ligand affinity for both alpha- (Gros et al., 1998) and beta-adrenoceptors (Cavali et al., 1996).

In addition, one other type of interaction, the hydrogen bonding between the chiral benzene β-hydroxyl of norepinephrine and the residues of the TM helix VI determines the stereoselectivity for adrenergic agonists and antagonists (Finch et al., 2005). Green et al. (1993) showed that mutations in the serine residue (T4.56) to isoleucine produced a fourfold reduction in the adrenoceptor affinity for epinephrine and norepinephrine. They suggested that the loss of binding affinity stemmed from the mutation-induced conformational change which prevented the proper functional interaction between the ligand and the receptor. Adrenoceptor types and subtypes show ligand selectivity for epinephrine and norepinephrine with respect to both ligand affinity and agonist potency.

Aside from the stereoselective nature of ligand/receptor interactions, there are specific ligand-binding residues unique to each receptor type (α and β) that confer receptor selectivity. There are ligand-binding residues which are unique to the receptor subtypes that also confer subtype selectivity (Hwa et al., 1995). Only two of the over 176 amino residues that constitute a receptor TM helix account for the α_{1A} ligand selectivity (Hwa et al., 1995). In addition, receptor residues distinguished by their location on the TM segments (I – VII) also confer agonist and antagonist specificity. Phenylalanine residues in TM segments IV and V have been found to play a role in general α_1 agonist-binding (Waugh et al., 2000). In contrast to agonist binding, unique residues found on TM segments III – VII have been identified to confer antagonist specificity. Waugh et al. (2000) showed that mutations in these residues (TM IV and V) impact the affinity

of the agonist phenylephrine on the α_{1A} receptor, but do not affect binding of the antagonist prazosin to the same receptor.

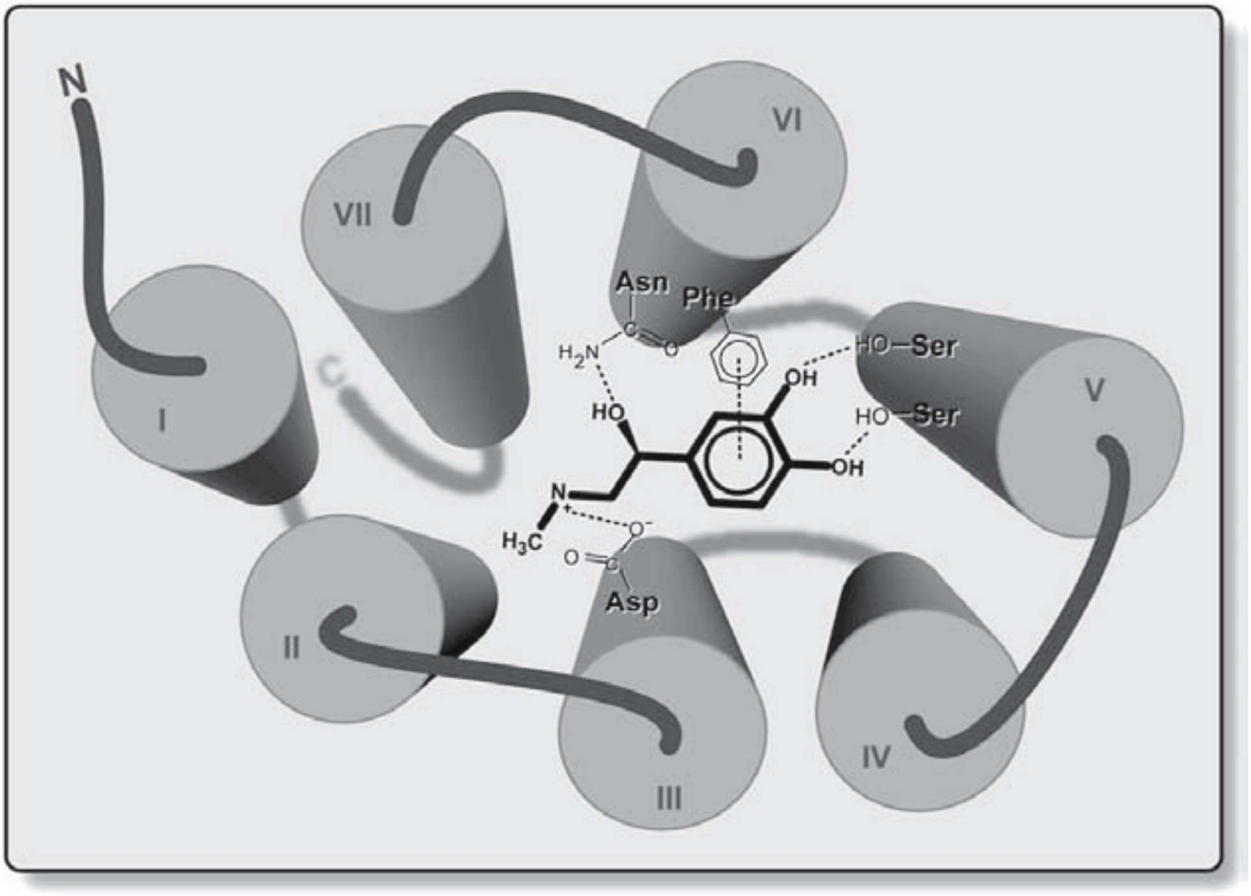


Fig 3. Generic representation of an adrenoceptor (seven transmembrane protein) displaying the functional interactions between the ligand, epinephrine and the receptor binding sites. From Perez (2005).

1.2.4. Adrenoceptors and functional effects

Adrenoceptors play a role in the regulation of many physiological functions including those involved in circulatory, metabolic, respiratory and central nervous system homeostasis (Medgett and Rajanayagam, 1984). Activation of adrenoceptors has been found to cause an increase in heart rate (Suita et al., 2015), dilation of bronchioles in the lungs, decreased gastrointestinal movement and blood vessel constriction (Tank et al., 2015; Suita et al., 2015). In the mammalian circulatory system, adrenoceptors have the crucial role of mediating vascular smooth muscle contraction. Depending on their location, catecholamines activate different adrenoceptors and have different physiological effects. For example, the effects of NE in the cardiac muscles through the activation of β receptors differ from the activation of these same receptors within smooth muscles in the peripheral vasculature. Activation of β adrenoceptors in the heart mediate increases in both cardiac rate and contractility, whereas stimulation of β adrenoceptors in the peripheral vessels mediate smooth muscle relaxation (Xiang and Kobilka, 2006).

Several studies have investigated the effects of the sympathetic neural transmitters in adrenoceptor activation (binding) and the mechanisms of receptor action (signaling transduction pathways) affecting the physiology of the cardiovascular system. Major categories of adrenoceptors have also been differentiated based on the class of G-protein to which they bind. Each class of G-protein has been defined based on the structure and function of the alpha subunit the TM helix couples (figure 2). In mammals, the alpha adrenoceptor can couple with three different alpha subunit types which make up the trimeric G-protein coupling unit, which include the G(s), G(q) and G(i) alpha subunits (Hildebrandt, 1997; Albert and Robillard, 2002).

Adrenoceptors have been shown to activate pathways specific to the alpha G-protein subunit they bind. The functional significance of the G-protein subunit heterogeneity is related to the different mechanisms by which the G-proteins transduce the effect of neurotransmitters and the cellular changes occurring through the signaling transduction pathways (Hildebrandt, 1997). With regards to alpha adrenergic receptors, activation of α_1 receptors activates the G_q coupled alpha subunit pathway, whereas activation of the α_2 activates the G_i coupled alpha subunit pathway, each of which activate different downstream signaling pathways (Figure 2). Stimulation of the α_1/G_q receptor G-protein complex activates the enzyme phospholipase C (PLC) in a cascade of events which catalyze the hydrolysis of phosphatidylinositol 4, 5-bisphosphate causing intracellular Ca^{2+} release and the activation of protein kinases (e.g. PKC). Physiologically, this produces depolarization of vascular smooth muscle and vasoconstriction (Mori et al., 2015). In contrast, stimulation of the α_2/G_i receptor G-protein complex mediates two functional effects: first, the inhibition of adenylyl cyclase (and its catalytic conversion of ATP to cAMP) which decreases intracellular Ca^{2+} release in vascular smooth muscle cells, causing vasoconstriction (Cavarape et al., 2003). Secondly, a negative feedback loop is activated through the presynaptic α_2/G_i complex which inhibits (and/or regulates) calcium release within the sympathetic nerve endings, inhibiting the release of neurotransmitter from the vesicles (Hernandez et al., 2007). The functional significance of the pre-junctional α_2 activation is to decrease the neurotransmitter release and regulate sympathetic-mediated vascular tone (Hernandez et al., 2007). Figure 2 summarizes the adrenoceptor subtype classification and their mechanisms of action.

1.2.5. Pharmacology of adrenoceptors and their functional effects

Several techniques have been very useful in characterizing the function of adrenoceptor types and subtypes in vascular pharmacology. Pharmacological differences in vasoconstrictor responses to sympathetic nerve stimulation have well demonstrated the role of the adrenoceptors in vessels. Studies using selective receptor blocking drugs have suggested the predominant involvement of post-junctional α_1 adrenoceptors in the nerve-mediated vasoconstrictor responses since the responses were seldom affected by selective α_2 blockers. In proximal segments of the rat tail artery, Medgett and Langer (1984) found significant reduction in neurogenic-mediated vasoconstrictor responses due to the selective α_1 antagonist prazosin. However, these responses were not significantly affected by the selective α_2 antagonist idazoxan (Medgett and Langer, 1984; Medgett and Rajanayagam, 1984). In rat and porcine pulmonary arteries, contractile responses to sympathetic nerve stimulation were significantly inhibited by selective α_1 antagonists, prazosin and WB4101, as well as the neuronal Na^+ channel blocker tetrodotoxin (Duggan et al., 2011), whereas cocaine enhanced these contractile responses (Duggan et al., 2011). Studies have suggested that the contractile function of VSMC is primarily dependent on α_1-adrenoceptor activation whereas postganglionic depolarization is dependent on α_2 activation (Brock et al., 1997; Jobling et al., 1992). The application of other molecular techniques including receptor protein-site mutagenesis and transgenic (receptor) knock-out studies extend the functional findings of the adrenoceptors of previous pharmacological approaches. Targeting the genes responsible for the expression of receptor types has also been a valuable approach to determine the physiological responses they affect.

1.2.6. Sympathetic innervation of the caudal vasculature

The main ventral artery in the rat tail is densely innervated with noradrenergic nerve plexus (Sittiracha et al. 1987) and this density appears to decrease down the tail, from proximal to distal end (Jobling and McLachlan, 1992). For this reason, the proximal segment of the rat tail artery has been very instrumental in studies that aim to characterize the function of alpha-adrenoceptors and provides a unique opportunity to study α-adrenoceptor activation in normal and disease states. Studies have demonstrated alpha-adrenoceptors in the rat tail artery are responsible for mediating the effects of neuronally released noradrenaline (Tanaka et al., 2004). Histochemical studies reveal the abundance of $alpha_{1A}$ and $alpha_{1D}$ adrenoceptor subtypes in the proximal segment of the main ventral tail artery in rats (Kamikihara et al., 2007). Similarly, other histochemical studies have demonstrated the presence of α_1-adrenoceptors on the vascular smooth muscle of the tail artery in contrast to the α_2 receptors which are predominantly present on the endothelium of vascular tissues (Kamikihara et al., 2007). These findings have been corroborated by physiological studies in control animals revealing the functional differences of these alpha receptors. For example, the electrophysiological studies by Jobling et al. (1992) demonstrated that contractile responses in rat caudal arteries were antagonized by α_1 blockers (e.g. prazosin) but not α_2 blockers. However, the neuronal alpha depolarization (NADs) were only significantly antagonized by the α_2 blocker (e.g. idazoxan) but not the α_1 blockers.

All the same, there appears to be no data on the effects of inflammation on adrenoceptor function and their associated vascular nerve-mediated contractile responses.

1.3. Inflammation in the cardiovascular system

Inflammation is a protective biological response of tissues in the body to foreign stimuli such as pathogens or damaged cells. This biological response, also known as the inflammatory response, involves the detection of a wide variety of foreign particles and is characterized by signs of swelling due to increased vascular permeability, pain, redness, heat and loss of function (Rather, 1971). Controlled inflammation is necessary for immunological function as immune regulatory cells are constantly interacting with foreign bodies in the system to regulate pro-inflammatory effector cells (Jiminez et al., 2015). Hence, the purpose of this response is to eliminate the foreign bodies and return functional normality to the system. The inflammatory process involves many cells including immune cells (e.g. macrophages, T-cells and neutrophils), vascular tissues, and membrane mediators which interact with foreign bodies. Although this biological response is normal and beneficial for the organisms in regulating infection and injury, excessive and persistent inflammatory response could contribute to a variety of inflammatory diseases. Furthermore, the duration of the inflammation response is also important in characterizing vascular diseases associated with chronic inflammation persisting for weeks, months or longer, as well as acute episodes only lasting hours mediated by increased movement of plasma and leukocytes from blood to the interstitial space (Jimirez et al., 2015).

1.3.1. The inflammatory response

The innate and adaptive (humoral) systems constitute the two important lines of defense of the immune system (Warrington et al., 2011). Both immune systems, are involved in

the detection of foreign particles and the inflammatory response. The innate system is the first immunologic and non-specific line of defense and plays an important role in the rapid initiation of a response. Here, the presence of a foreign particle is detected by the free circulating monocytes and leukocytes. Adaptive immunity, on the other hand is an antigen-dependent and antigen-specific response that enables rapid reactivity in the event of pathogen reoccurrence or prolonged exposure. In either line of defense, this detection is made possible by adhesion molecules, expressed on the surface of leukocytes. These adhesion molecules interact with and recognize the particle-associated molecular patterns (PAMPs) displayed on the foreign particles and initiate a reaction necessary to trigger the inflammatory response. In vascular tissues, these adhesion molecules are expressed on the innermost endothelial cells. Binding to a "stimulus" or foreign particle activates the leukocytes in a cascade of events resulting in the release of cytokines which modulate the inflammatory process (Warrington et al., 2011). Several studies on inflammation have recognized some important pro-inflammatory cytokines involved in the cascade for the response in inflammation. Some of these are tumor necrosis factor-alpha (TNF-α), interferon gamma (IFNγ), interleukine-1, 6 and 8 (Calder, 2006; Dinarello, 1996). Several studies have consistently demonstrated a positive correlation between the expression of these cytokines and the inflammatory response, in conditions where the expression of such cytokines is upregulated during chronic inflammation (Marques-Rocha et al., 2015); the reason they are termed pro-inflammatory cytokines and why the expression of these cytokines is used as experimental and clinical predictors of inflammation.

Depending on the location of the inflammatory reaction, the response can be deemed localized or systemic. In localized inflammation, the inflammatory process is confined to a

certain organ or structure within the body. In systemic inflammation, however, the inflammatory reaction is general within the body. In systemic inflammation, the response is not confined to any particular tissue but involves the endothelium and other organ systems. This is possible where the stimuli or mediators of the inflammatory response are transmitted through the cardiovascular system by way of the circulating blood, affecting other organ systems. Essentially, in systemic inflammation, the response is everywhere, and may involve multiple organ tissues. All systems and especially the vascular tissues undergo inflammation to the existing stimuli. The cascade of events involving the expression of inflammatory markers (cytokines) plays an important role to (1) initiate the inflammatory response by activation of the innate immune system and (2) link both (innate and adaptive) mechanisms of the immune system in mediating the inflammatory response.

1.3.2. Inflammation and cardiovascular disease

The presence of foreign particles in blood triggers a controlled (protective) inflammatory response by initiating mechanisms in the vascular walls that mediate inflammation. Some of these mechanisms involve the expression of adhesion molecules (ICAMS and VCAMS) on the endothelial and smooth muscle cell surface, which promotes the recruitment of leukocytes, monocytes and macrophages to the sites of inflammation (Brown et al., 2001; Hirata et al., 1998). Chronic inflammation has been found to induce vascular endothelial and smooth muscle cell remodeling including collagen deposition which accentuates the onset of altered vascular function (Lee et al., 2015). In a study to investigate leukocyte/monocyte expression after TNF-α infusion in human umbilical vein endothelial cells (HUVEC), Pu et al. (2002) demonstrated endothelial cell modifications (i.e. that increased the

expression of VCAMs) which altered the cell-to-cell interactions and the function of the endothelium. Hence, chronic inflammation has been associated with the development of a range of both acute and chronic diseases. Evidence of inflammation in cardiovascular diseases is most often characterized by the increased expression of adhesion molecules and endothelial cell damage (Collie-Duguid and Wahle, 1996; Hirata et al., 1998) as well as the excessive production of pro-inflammatory cytokines (Dinarello, 1996) in the vascular tissue. The characteristic increase in blood flow and vascular permeability in states of inflammation also allows the increased movement of leukocytes and cytokines across the endothelium into the surrounding tissues (Calder, 2006). Such chronic responses cause excessive tissue damage and the aggregation of leukocytes and monocytes in surrounding tissues which maintains or amplifies the response. Researchers have linked the expression of inflammatory mediators to asthma (Henderson, 1992), type 2 diabetes and obesity. Elsewhere, the excessive productions of pro-inflammatory cytokines (TNF-α, IL-1, IL-6) occurring during chronic inflammation have been implicated in some disease states such as rheumatoid arthritis, acute respiratory syndrome, irritable bowel disease, Alzheimer's, peripheral artery disease (PAD) and atherosclerosis (Pande et al., 2014). As such, inflammation is well recognized for its important contribution in the pathophysiology of cardiovascular disease.

The release of these cytokines has been associated with the development of some cardiovascular diseases such as atherosclerosis and heart reperfusion injury. In the cardiovascular system, the role of the inflammatory processes in the development of vascular lesions is well documented. A recent meta-analysis conducted by Zhang et al. (2015) found a positive correlation between vascular intima media (endothelial layer) thickness and levels of

IL-6 in patients with cardiovascular disease. Similarly, Pande et al. (2014) demonstrated an association between TNF-α, a biomarker of systemic inflammation and the occurrence of peripheral artery disease. Pande et al. (2014) found significant elevations in the expression of the monocyte TNF-α genes in patients with peripheral artery disease; which exerted a negative impact on patients' cardiovascular health. Furthermore, evidence of hyperglycemia-induced activation of leukocytes in type 2 diabetic patients and its involvement in vascular damage (de Vries et al., 2014) suggests it plays a role in the inflammatory process in conjunction with other metabolic defects such as those associated with obesity.

The role of inflammation in cardiovascular diseases has increased research interest and activity in the field of cardiovascular function in those affected. Research in cardiovascular function has demonstrated that changes in vascular physiology and function stem from inflammation and are responsible in promoting vascular diseases such as atherosclerosis. Seemingly, endothelial dysfunction, a characteristic of vascular inflammatory diseases causes vascular stiffness and alters the contractility of smooth muscle cells in vascular tissues (Huveneers et al., 2015). Leal et al. (2015) found contractile hyper-responsiveness of atherosclerotic mice aortic rings to phenylephrine in apolipoprotein- (apoE) treated mice compared to the controls. Interestingly, studies assessing vascular changes in hypertensive animals have reported similar findings where endothelial dysfunction has been linked with a heightened contractile sensitivity and increased sympathetic reactivity (Mori et al., 2015) within vascular smooth muscle cells. These therefore, present a link between inflammation, vascular tissue (endothelial and smooth muscle cell) dysfunction and cardiovascular disease.

1.3.3. Inflammation and effector signaling pathways

The involvement of chronic inflammation at the onset and progression of many diseases including type-2 diabetes, obesity, atherosclerosis, and asthma is mediated by the expression of cytokines and adhesion particles in organ endothelial cells (Marques-Rocha et al., 2015). Pro-inflammatory mediators play a role in activating immune system cells in plasma and vascular tissues. This activation of leukocytes and macrophages dictates a ligand binding relation between the chemotactic cytokines and the receptors on the endothelial cells as well as the receptors elsewhere in the vascular tissues (Marques-Rocha et al., 2015). Monocytes, macrophages and leukocytes express varying types of toll-like receptors (TLRs) that participate in cellular activation; and the initiation of a wide spectrum of responses during inflammation (Akira et al., 2006; Marques-Rocha et al., 2015). Little is known about the signaling pathways by which the inflammatory process (i.e. cytokines) activate the immune cells and maintain the inflammatory process. The signaling pathways and regulatory mechanisms involved in vascular diseases as a result of chronic inflammation remain incompletely understood.

Nevertheless, some studies have linked the expression of pro-inflammatory cytokines with the activation of certain signaling pathways such as phospholipase C (PLC) and protein kinase C (PKC) to the initiation of the inflammatory response in various disease states. Olofsson et al. (2006) previously reported the involvement of CD137, a member of the TNF superfamily in atherosclerosis and the onset of inflammatory plaques. Its expression in cardiomyocytes, vascular endothelial and smooth muscle cells suggest that it promotes atherosclerotic plaque development (Olofsson et al., 2008; Tedgui & Mallat, 2006). Its interaction with CD137 ligand has been implicated in the activation of the TNF pathway and the development of

atherosclerosis (Yan et al., 2013; Olofsson et al., 2008; Tedgui & Mallat, 2006). Recent work suggests that CD137-CD137L interaction induces activation of PLC signaling pathway in human umbilical vein endothelial cells (HUVEC) (Yan et al., 2013). This PLC signaling pathway commonly known for the synthesis of diacylglycerol (DAG) and inositol triphosphate (IP3), plays an important role in cellular calcium regulation and sympathetic nerve-mediated smooth muscle contraction. In HUVEC, CD137-CD137L interaction produced a concentration-dependent increase in DAG and IP3 levels indicating the activation of the PLC signaling pathway by the TNF-α mediator (Yan et al., 2013). Others have demonstrated the involvement / activation of the PLC signaling pathway in other metabolic inflammatory diseases such as diabetes. For example, the observation of enhanced insulin release in the pancreas by Suzuki et al. (2011) suggested a direct link between interleukin-6 (IL-6) expression and insulin resistance because IL-6 receptors were overtly expressed in the murine pancreatic β cells during insulin resistance.

Therefore, the role of cytokines and their interaction with specific receptors has been implicated in the onset and progression of cardiovascular diseases; however, their role in adrenoceptor-mediated vascular function (contraction and dilation) has yet to be explored.

1.4. Vascular smooth muscle cells

Smooth muscle cells make up the thickest layer of vascular tissue, the tunica media. Depending on the vessel, the media could consist of several layers of SMCs ranging from one to four or more layers. Within each layer, the smooth muscle cells are arranged in a helical fashion, superimposing each other at angles contributing to the ring structure of the vessel (Garland & Angus, 2006). For decades, the contractile response of smooth muscle cells has

been studied in preparations of large arteries using the spiral-cut technique. This spiral-cut technique has been used commonly for its advantage that changes in smooth muscle cell length were amplified due to the mechanical arrangement of the VSMCs (Garland & Angus, 2006).

1.4.1. Electrical properties of VSMCs

The behavior of vascular smooth muscle cells is controlled by the activity of the sympathetic postganglionic nerves (Tank & Lee Wong, 2015). The activation of the sympathetic nerves that innervate the smooth muscle cells is dependent on intermittent bursts of varying amplitude and frequency (Stjärne, 2000). In particular, this burst discharge and the associated excitatory junctional potentials (e.j.p.) are responsible for the arterial smooth muscle cell contractions, causing vasoconstriction. On the graded potential principle, single e.j.p.s are not enough to produce a threshold potential to activate the sympathetic nerves but a summation of single e.j.p.s fire at threshold. At threshold, action potentials cause the sympathetic nerves to release their neurotransmitter contents from the varicosities (in distal axons) into the "synaptic cleft". Interestingly, the bursts of e.j.p.s have been associated with the "quantal" release of neurotransmitter. Furthermore, within a certain physiological range, there is linear relation between vascular contractile response and frequency of stimulation (Bevan, 1978). The greater the frequency of firing potentials (pulse), the greater the amount of varicosities that get activated and release their noradrenaline (NA) contents in response, and the greater the SMC inotropic (contractile) effect. During sympathetic activation, whether NA is released from a small percentage of varicosities or small quantities from all the varicosities remains unclear. However, the intermittent release of noradrenaline in association with the pulse of activation is evident in amperometric studies (Stjärne, 2000). Furthermore, the varying amplitudes and

frequencies that produce sympathetic e.j.p.s also seem to have different thresholds in different species. In rats for example, low-firing frequencies have been estimated as low as 0.5Hz and high-firing as high as 50Hz. Duggan et al. (2011) were able to demonstrate EFS-evoked smooth muscle contractile responses in rat pulmonary arteries between 0.6 – 40 Hz. This frequency range has been reproducible in the rat femoral artery between 5 – 30Hz (Zacharia et al., 2004).

1.4.2. Mechanical properties of VSMCs

The sympathetic release of noradrenaline and adrenergic receptor activation mediate the contractile responses in vascular tissues. Alpha-adrenoceptor activation (i.e. PLC and adenylyl cyclase signaling pathways) induces changes in cytosolic concentrations of calcium through the release of calcium from the cell's internal stores (e.g. sarcoplasmic reticulum). This is in contrast to the voltage-dependent influx of calcium from extracellular stores triggered by ATP for example. Here, this calcium release (and/or influx) leads to an increase in transient intracellular calcium, and activates the myosin-actin contractile machinery (Garland & Angus, 2006). In the smooth muscle cells, the calcium-bound calmodulin complex activates the myosin light chain kinase (MLCK) enzymes to phosphorylate the myosin filaments. Hence, the active calcium-dependent phosphorylated myosin filaments interact with the actin filaments (known as the sliding filament mechanism) to form cross-bridges. The cross-bridges cause contraction of the myosin-actin complex, and ultimately contraction of the entire smooth muscle tissue. The functional significance of the myosin-actin interaction in the vasculature, therefore, is the contraction and relaxation of the smooth muscle cells which permits an increase or decrease in resistance in vessels, respectively, and regulate blood flow.

Not surprising then, changes in calcium handling proteins can functionally alter the smooth muscles ability to contract and relax. In vascular diseases, the expression and function of calcium handling proteins (pumps, exchangers and channels) involved in smooth muscle contraction and relaxation are often altered (Ewart et al., 2014; Lipskaia et al., 2014).

1.4.2. Pharmacology of vascular smooth muscle

In vitro, vascular tissue segments retain much of their neuronal and mechanical function, which allows the assessment of the effects of vasoactive agents in isolated target tissue (Garland and Angus, 1996). Vasoactive agents such as agonists and antagonists of adrenoceptors present the unique opportunity to study the vasomotor properties of smooth muscle cells. Phenylephrine is a selective α_1-adrenoceptor agonist which produces concentration-dependent contractions of vascular smooth muscle cells. The vascular concentration-dependent contractile response to PE are abolished by the presence of prazosin, a selective α_1-adrenoceptor antagonist (Duggan et al., 2011). EFS-contractile response studies have demonstrated that the sympathetic contractile response mediated by the NA release is also affected by antagonistic action. Selective α_1-adrenoceptor antagonists have been shown to abolish the sympathetic-mediated responses. Sympathetic-mediated responses are significantly decreased (or abolished) by selective α_1-adrenoceptor blockers prazosin (Docherty, 2014; Duggan et al., 2011, Zacharia et al., 2004), terazosin and doxazosin (Mathur et al., 2014; Persson et al., 1998). Similarly, the α-adrenoceptor blocker WB4101 has also been found to significantly inhibit field stimulation contractile responses (Duggan et al., 2011). Docherty (2014) found greater affinity of prazosin for the α_{1A}- and α_{1D}-adrenoceptors, subtypes previously demonstrated to mediate the contractile response in proximal segment of the rat

tail artery. Conversely, the selective α_2-adrenoceptor blocker rauwolscine has been found to have endothelium dependent effects, to either decrease EFS-mediated contractions (Pagan et al., 2012) or to augment the nerve-mediated contractile responses (Martinez et al., 2009) in the presence of functional endothelium in large arteries. Furthermore, noradrenaline reuptake antagonists potentiate the nerve-mediated contractile responses to EFS stimulation. Cocaine, a common NA reuptake inhibitor has been to found to enhance the field stimulation frequency-dependent responses (Docherty, 2014; Duggan et al., 2011). In the presence of cocaine, the predominant subtypes (α_{1A}- and α_{1D}-adrenoceptors) involved in VSM nerve-stimulation-evoked contractile responses were found to be more sensitive to noradrenaline (Zacharia et al., 2004). Altogether, studies have posited differences in the frequency ranges (low, 0.5-5 Hz and high, 10-40 Hz) where the nerve-mediated contractile responses are most sensitive to the inhibitory action of the various antagonists but these frequency-dependent differences remain inconclusive.

1.5. Rationale

Physiological and pharmacological studies have provided great insight on the function of adrenoceptors and increased the understanding of how these receptors function in different physiological systems. There is ample evidence that alterations in sympathetic nerve function are implicated in a variety of pathophysiological states, and cardiovascular diseases in particular (Chistiakov et al., 2015; Derosa et al., 2007; Topal et al., 2006; Tesfamariam et al., 1992; Shafi et al., 1989). Furthermore, a body of research has suggested that changes in adrenoceptor structure and regulation may also play a role in the development of certain cardiovascular diseases (Mathur et al., 2014; Amiya et al., 2014; Pagan et al., 2012). With regards to α-

adrenoceptors in particular, changes in functionality is related to vascular diseases associated with changes in vascular contractile regulation and vascular tone.

There is now some understanding of how changes in vascular tissue due to inflammation may impact the smooth muscle cells and relate to the development of vascular diseases such as atherosclerosis and high blood pressure. Nevertheless, it is still unknown how the processes that modulate systemic inflammation may affect the adrenoceptors and the associated vascular sympathetic function. Therefore, because drugs targeting these AR's are very important and are widely used in clinical therapy, understanding the changes in adrenoceptor structure (/distribution) and function may elucidate understanding to tailor clinical therapy for individuals affected by vascular inflammation. The present study aimed to investigate the effects of inflammation on vascular alpha-adrenoceptor-mediated contractile responses and the neurogenic-mediated vascular function.

1.6. Hypothesis

We believe that chronic inflammation would affect the nerve-mediated contractions of blood vessels in rat tail arteries. We have reason to believe that pro-inflammatory cytokines can activate smooth muscle cells through activation of the phospholipase C signaling pathway. If this occurred, nerve-mediated contractions would be expected to be greater in tail arteries from inflamed animals compared to the control rats. Increased vascular tone has been evident in inflammatory-mediated vascular diseases as a result of increased sympathetic activity and the greater release of the neurotransmitter noradrenaline. Since sympathetic nerve activity leads to the activation of adrenergic receptors, the increased outflow of NA would be expected

to produce larger α-adrenoceptor-mediated smooth muscle contractions in inflamed animals compared to control animals. We expected that NA reuptake blockers would produce a larger potentiation of the nerve-mediated vascular contractions in inflamed animals in comparison to the controls.

We also assessed the possibility that a differential distribution (i.e. localization and density) of α-adrenoceptors existed in the rat tail artery from inflamed and control animals. In addition, functionally, we expected the nerve-mediated contractile responses to activation of α_1- and α_2-adrenoceptors to be altered in inflamed animals compared to the control animals.

1.7. Objectives

The present study aimed to investigate the sympathetic nerve-mediated contractile responses resulting from alpha-adrenoceptor activation in blood vessels of animals subjected to chronic inflammation in comparison to healthy animals. In order to investigate the above, the present study will:

i. Establish a rat model for chronic systemic inflammation. Compare the levels of TNF-α (a pro-inflammatory cytokine and marker for inflammation) over time in the plasma of treated and control animals using western blots to confirm systemic inflammatory response.
ii. Perform tension-studies to compare vascular nerve-mediated smooth muscle contractile responses to EFS.
iii. Assess the pharmacological characteristics of post-junctional α_1- and α_2-adrenoceptors using alpha-adrenoceptor antagonist action on nerve-mediated

vasoconstriction to compare adrenoceptor type contributions to SMC contractile responses.

iv. Compare the blocking properties of the adrenoceptor antagonists in the presence or absence of the NA uptake blocker, cocaine to assess any differences in NA adrenoceptor activation (pathway) in vascular contractile responses in treated vs control animals.

v. Employ immunofluorescence techniques to compare the distribution of alpha adrenoceptors and the sympathetic enzyme, tyrosine hydroxylase in the vascular tissue segments of treated and normal animals.

vi. Compare blood pressure changes over time, to verify normal blood pressure in the normotensive rat model. Compare localized inflammation by measuring ankle and paw volumes.

2.0. Materials and methods

2.1. Animals

All procedures on animals were performed in accordance with the guidelines and regulations of the Canadian Council on Animal Care, with the approval of the Institutional Animal Care Committee of Memorial University of Newfoundland. Male Sprague Dawley rats (5-6 weeks, 200-300 g) were housed in plastic cages in a temperature-controlled environment (22+/- 2 ^{O}C) on a 12:12h light/dark cycle. Animals were allowed access to regular chow and tap water, *ad libitum*.

2.2. Fine chemicals

Isofluorane was purchased from Halocarbons Products Corporation (USA). Buprenorphine was purchased from Schering Plough Ltd (UK). Heparin sodium was obtained from SoloPak Laboratories Inc. (USA) and Sigma-Aldrich Inc. (USA). Cocaine and tetrodotoxin were supplied by Sigma-Aldrich Inc. (USA). Research Biochemical International (USA) supplied rauwolscine hydrochloride, prazosin hydrochloride, WB4101, phenylephrine hydrochloride and methylcholine hydrochloride. Ketamine hydrochloride was supplied by Bimeda-MTC Inc. Xylazine hydrochloride was supplied by Bayer Health Care Inc. α_1 and α_{2b}-adrenergic receptor antibodies were purchased from Abcam Inc. (Toronto, ON). Anhydrous ethyl alcohol was obtained from Commercial Alcohols and hydrochloric acid was obtained from Fischer Scientific.

2.3. Animal model: chronic (systemic) inflammation

Systemic inflammation was established similar to the methods used by Schopf et al. (2006) by intradermal injection of Complete Freund's adjuvant (CFA; heat killed *Mycobacterium butyricum* suspended in mineral oil). Animals were anaesthetized with isofluorane (induced at 5% in 100% oxygen; maintained at 1.5-2.0% in 100% oxygen) delivered using masks. Left paws were surgically prepped (1: Betadine; 2: 70% Alcohol; 3: Chlorhexidine). CFA (0.05 - 0.07 ml) was injected intradermally in the hind left footpad of treatment animals. Sham-operated animals received equivalent volumes of saline. Flumazine was applied topically at the injection sites after injection to prevent infection.

Animals were then returned to cages and maintained on heat pads during recovery until they regained consciousness (physical activity). Buprenorphine (0.03 mg/kg; 0.5 mg/mL in normal saline) was administered subcutaneously immediately following surgery and at q12hr in CFA-treated animals for three weeks.

2.4. Measurement of parameters

Blood Pressure.

Blood pressure (BP) of animals was measured weekly (for three weeks) using the non-invasive tail cuff method (plethysmography, IITC Life Science warming chambers) in wake animals from Day 0 (prior to inflammation). Before BP recording, animals were allowed to acclimatize in a warming chamber equipped with fans (IITC Life Science Warming Chambers; internal temperature set at 30^0C) for half an hour. Systolic BP readings were recorded with a Bonwin computer software.

Plasma samples

Blood samples were collected weekly for three weeks, starting on Day 0 before injection. Animals were anaesthetized with isofluorane (induction: 5% isoflurane, 100% oxygen; maintenance: 2.5% isofluorane, 100% oxygen) and placed on a heating pad. Blood (0.5-0.7 ml) was collected from the left or right tail veins using heparin-coated syringes. Blood samples were then centrifuged at 15,000 x g rpm (Legend Micro 21, Thermo Scientific) for 15 minutes at 4^0C. Plasma was separated, transferred to labeled tubes and stored at -80^0C for analysis of systemic inflammation.

Body weight and paw assessment.

Body weight, ankle and paw volume were measured in 1-2 day intervals, starting on Day 0 for three weeks. Animals were weighed using a regular digital weight scale. Ankle and paw measurements (left and right) were obtained using digital calipers to assess the inflammatory response. Inflammation was also assessed with a water displacement method of the ankle and paw (left and right). The weight of the displaced volume of water indicated the volume of the animal's foot (ankle and paw, combined).

2.5. Experimental preparation for Force-tension studies

2.5.1. Buffer solution

Krebs buffer was prepared from a mixture of two solutions of the following composition (mM) similar to Duggan et al. (2011): 130 NaCl, 4 KCl, 1.2 $MgCl_2.6H_2O$, 2.5 $CaCl_2.2H_2O$, 12.5 $NaHCO_3$, 1.2 KH_2PO_4 and 0.1 EDTA. A solution of high potassium (80mM) was also prepared where equimolar levels of sodium were replaced with potassium. Media solutions were gassed with 95% O_2 and 5% CO_2 at all times and maintained at 37^0C in a water bath.

2.5.2. Animals

Following the three week monitoring period, animals were sacrificed. Rats were anaesthetized with a mixture of 20 mg/ml Rompun and 50 mg/ml Ketalean (1:1 ratio) by intraperitoneal injection (i.p.). To induce full anesthesia, the dosage was adjusted while monitoring animal reflexes by toe-pinching. A midline incision from the xiphoid process to the neck provided access to the thoracic cavity. Animals were exsanguinated from the left ventricle, and the ventral caudal arteries were quickly extracted and placed in Krebs buffer.

2.5.3. Ventral tail artery

The proximal end of the ventral tail arteries was isolated. Helical strips (segments approximately 1.5 – 2 cm) were dissected in Krebs buffer gassed with 95% oxygen and 5% carbon dioxide at all times (pH: 7.4). Helical strips were then mounted on holders with rings of platinum electrodes, connected to an electric field stimulation system (Grass S88 stimulator). Tissues were placed in organ baths containing 20 ml Krebs buffer (gassed with 95% O_2 and 5%

CO_2, maintained at 37^0C and 7.4 pH) and connected to a force displacement transducer. Vascular contractile responses were recorded on a PC-based data acquisition system, AcqKnowledge 3.9.1 (Biopac Systems Canada Inc.).

2.6. Experimental protocols for Force-tension studies

Tissues were subjected to a resting preload tension of 200 mg (pre-determined by concentration-dependent force-tension curves to phenylephrine) and allowed to equilibrate in baths for 30 minutes. Contractile responses of tissues to electric-field stimulation were then studied. Effects of pharmacological agents to field-stimulated contractions at various frequencies were investigated. Tissues were washed three times with Krebs buffer every half hour and allowed to stabilize initially for 40-45 minutes.

Samples of the caudal artery (approximately 5-10 mm, buffered at pH 7.4) from animals were also fixed in 10% formalin and embedded in paraffin blocks for immunohistochemical studies. Sections were cut at 4-5 microns, placed on slides as needed and preserved at 4^0C for staining.

2.6.1. Electric Field Stimulation

Sympathetic nerves were stimulated with electric field stimulations similar to the method of Duggan et al. (2011). Frequency-dependent contractile responses were performed by exposing isolated tissues to electric fields of varying frequencies. A Grass Stimulator delivered electricity (100 mA, 50 V, 0.5 ms) through the platinum electrode rings on the tissue holders.

Tissues were stimulated at 50 V with a 0.5 ms pulse duration at varying frequencies (1.25-40 Hz). After equilibration, the tissues were subjected to three blocks of stimulation in the presence or absence of a pharmacological agent and/or vehicle. A total of nine pharmacological groups were tested (See Table I in appendix). Tissues were washed with Krebs solution after each stimulation block and allowed to equilibrate for 20-30 minutes.

Tissues were assigned to pharmacological groups on an *ad hoc* basis. The presence of functional endothelium was randomly assessed in tissues with methacholine (10^{-6} M) after a contractile response to phenylephrine (10^{-6} M). Furthermore, the neurogenic origin of the contractile responses was verified with field stimulations by addition of tetrodotoxin (TTX, 10^{-7} M) to the bath. Maximal contractile responses were produced in all tissues after field stimulated contractions with a solution of high potassium (80 mM) for normalizing of responses.

2.7. Assessment of systemic inflammation

TNF-α determination

Blood samples (collected weekly) of five randomly selected animals per group were analyzed for tumor necrosis factor-alpha (TNF-α) using the enzyme-linked immunosorbent assay (ELISA) technique. The ELISA protocol was provided with the Rat TNF-α ELISA Deluxe kit (Biolegend, San Diego. Ca). Immunohistochemical (IHC) techniques were also used to assess the expression of ionized calcium adapter binding molecule-1 (Iba-1) in paraffin-preserved vascular tissues. IBA-1 is specifically expressed in macrophages and microglial cells and is particularly upregulated when macrophages are activated systematically.

ELISA assessment of TNF-α in flash frozen plasma samples was performed as directed by the manufacturer (Biolegend, San Diego, CA, USA). Plate wells were coated with 100ul of mixed capture buffer solution (capture antibody in coating buffer) and incubated overnight at 4^0C. Plasma samples were thawed overnight at -20^0C from -80^0C. On the following day, plates were washed (four times) with wash buffer (0.05% tween20 in PBS). After wash, plates were blocked with 200µl 1X assay diluent in Phosphate-Buffered Saline (PBS; 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl in distilled water to 1000 ml) for one hour at room temperature on a shaker with circular orbit. Plates were washed again (four times) with wash buffer. Standard solutions were prepared as follows: 100ul of reconstituted standard (1:100 dilution of stock in 1X assay diluent). Six, two-fold serial dilutions of the top standard were performed. 1X assay diluent served as a zero standard. 50µl of Matrix A was loaded unto standard dilution wells; whereas 50µl of 1X assay diluent was loaded unto sample wells. Thereafter, 50µl of standard dilutions and plasma samples were added to the appropriate wells and incubated on shaker at room temperature for 2 hours. Plates were again washed (four times) with wash buffer. 100µl of diluted detection antibody (1:200 in assay diluent) was added to wells and incubated on shaker at room temperature for one hour. Plates were again washed (four times) with wash buffer. Thereafter, 100µl of Avidin-HRP diluted solution (1:1000 in assay diluent) was loaded into wells and incubated on shaker at room temperature for 30 minutes. Plates were washed (five times) allowing 1 minute per wash. Wells were then incubated in the dark with 100µl of freshly mixed TMB substrate (5.5 ml Solution A and 5.5 ml Solution B) for 25 minutes. 100ul stop solution was quickly added to the wells and plates read within 15 minutes at 450-570nm absorbance.

2.8. Immunofluorescent staining

α_1 & α_{2b}- adrenoceptors

Immunofluorescence (IF) was used to assess the expression and localization of alpha adrenoceptors and the sympathetic nerve neurotransmitter enzyme tyrosine hydroxylase (TH co-localized with α_{-2b} adrenoceptor) in the smooth muscle and endothelium of rat tail artery. IF staining was performed similar to the methods of Daneshtalab et al. (2010). Slides were deparaffinized in xylene (x2) and rehydrated in series dilutions of ethanol. Afterwards, endogenous peroxidase was blocked by incubating in 3% hydrogen peroxide for 15 minutes. Samples were then blocked with 10% horse serum and 0.1% triton x in phosphate-buffered saline (PBS) for 30 minutes in a humid chamber. Antigen retrieval was achieved by exposing samples to 1% Sodium Dodecyl Sulphate (SDS) at room temperature for 5 minutes. Sections were washed in PBS on a tilt tray after every step for 3 minutes. After antigenic exposure, samples were incubated overnight at 4^0C with either a primary rabbit polyclonal alpha-1 (1:75 in 2% horse serum; Abcam, Toronto, ON.) or mouse monoclonal alpha-2b antibody (1:75 in 2% horse serum; Thermoscientific, Burlington, ON) and rabbit tyrosine hydroxylase antibody (1:750 in 2% horse serum; Abcam, Toronto, ON). 2% horse serum (HS) was prepared in PBS.

The following day, the sections were washed sequentially with Tris-Buffered saline with Tween20 solution (TBST; 20mM Tris Base, 150mM NaCl and 50mM tween20) for 10 minutes. After primary antibody removal, the respective secondary antibody was added: 1:300 against cy2 goat anti-rabbit, 1:300 against cy2 goat anti-mouse and 1:250 against cy5 goat anti-rabbit in 2% HS for 30 minutes respectively at room temperature. Secondary antibody incubation and

washes were completed in an aluminum-covered humid chamber and tilt trays to limit access of light (in dark). Afterwards, secondary antibodies were washed four times with TBST for 10 minutes. Sections were flooded with 50% glycerol and covered with coverslips tight and bubble-free. Edges were sealed with transparent nail polish to hold coverslips in place.

IBA-1 staining

A similar IF staining protocol was used for IBA-1 staining, in the vascular tissues. After deparaffination, sections were hydrated through graded alcohols to water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes in a humid chamber and rinsed with 1X PBS. Antigen retrieval was achieved by incubating sections in a lid-covered dish containing citrate buffer placed in a steamer (at 95-100^{0}C) for 30 minutes. Afterward, the dish was removed from the steamer and allowed to cool at room temperature for approximately 20 minutes. Vessels were rinsed with PBST (3% triton x in 1X PBS). Sections were blocked with 10% normal goat serum (NGS, in Tris-Buffered saline) in a humid chamber at room temperature for 2 hours. Sections were again rinsed with PBST. After blocking, sections were incubated overnight at 4^{0}C with primary rabbit polyclonal Iba-1 antibody (Abcam, Toronto, ON. CA) prepared as 1:1000 dilution in 2% NGS.

The following day, sections were rinsed (twice) and washed (four times) in 1X PBS on a tilt shaker at room temperature. Then, sections were incubated with secondary antibodies against rabbit Iba-1: cy2 goat anti-rabbit (1:200 in 2% NGS) and 4’ 6-diamidino-2-phenylindole (DAPI; 1:1000 in 2% NGS) for 30 minutes in the dark (aluminum-covered humidifier). Afterward, sections were rinsed (twice) and washed (four times) with 1X PBS at room temperature on a tilt

shaker for 10 minutes. Sections were drained and dried with absorbent paper (kimwipes) and flooded with 50% glycerol (glycerol in 1X PBS). Section slides were covered with coverslips and sealed with transparent nail polish.

Slides were imaged at 20X magnification using confocal microscopy and analyzed with Fluoview software (Olympus Fluoview, FV100). Sections from five slides of different animals per group were stained and imaged. The same laser parameters (laser intensity, transmitted light and offset) were used to image all sections for each IHC staining group (1. Alpha-1 and 2. Co-localization of alpha-2b and tyrosine hydroxylase).

2.9. Analysis

2.9.1. Data measurements and calculations

Mean systolic BP, weight, ankle and paw volumes were calculated as an average of three data points. EFS contractile response curves were analyzed for area under the mechanogram. Areas under the response curve were calculated as a total area sweep under the curve from $baseline_i$ to $baseline_f$. All data points were normalized as a percentage of the area (30 seconds after rise) to the high potassium-induced maximum contractions.

2.9.2. Statistical analysis

All measurements (area under the mechanogram) for the electric field-evoked contractions were calculated as a percentage of the contractile response to the high potassium solution of the respective tissue to normalize the data. Analysis were completed with SigmaPlot 12.0 (SPSS Inc., USA). Unpaired t-test of samples was carried out for the contractile responses

(control) in the absence of test drugs or vehicle (area under the mechanogram) between the CFA and Saline groups. Two-way analysis of variance (ANOVA) were carried out for comparisons of pharmacological effect within and between CFA and Saline groups, across frequency stimulations. The data are based on replications from at least 7 individual animals. Unpaired t-test was also carried out for animal systolic BP, weight, and ankle and paw size.

3.0. Results

CFA and saline groups of rats had similar mean blood pressures prior to treatment. There were no significant differences in the mean systolic blood pressure of saline-treated and CFA-treated rats over the period of 4 weeks (Table 1).

Baseline weights were not significantly different between saline and CFA groups. Animals treated with CFA had a significantly lower weight compared to their saline counterparts, over time (Figure 4).

Table 1. Mean systolic blood pressure of rats treated with saline or CFA over time. Mean ± standard error of mean. *P*>0.05 in all comparisons. N = 25 (in each condition)

	Week 1 (baseline)	Week 2	Week 3	Week 4
Saline-treated (mmHg)	167.7 ± 4.8	162.7 ± 6.0	159.4 ± 3.9	157.2 ± 4.8
CFA-treated (mmHg)	166.5 ± 5.4	166.4 ± 6.2	163.6 ± 4.9	158.5 ± 3.7

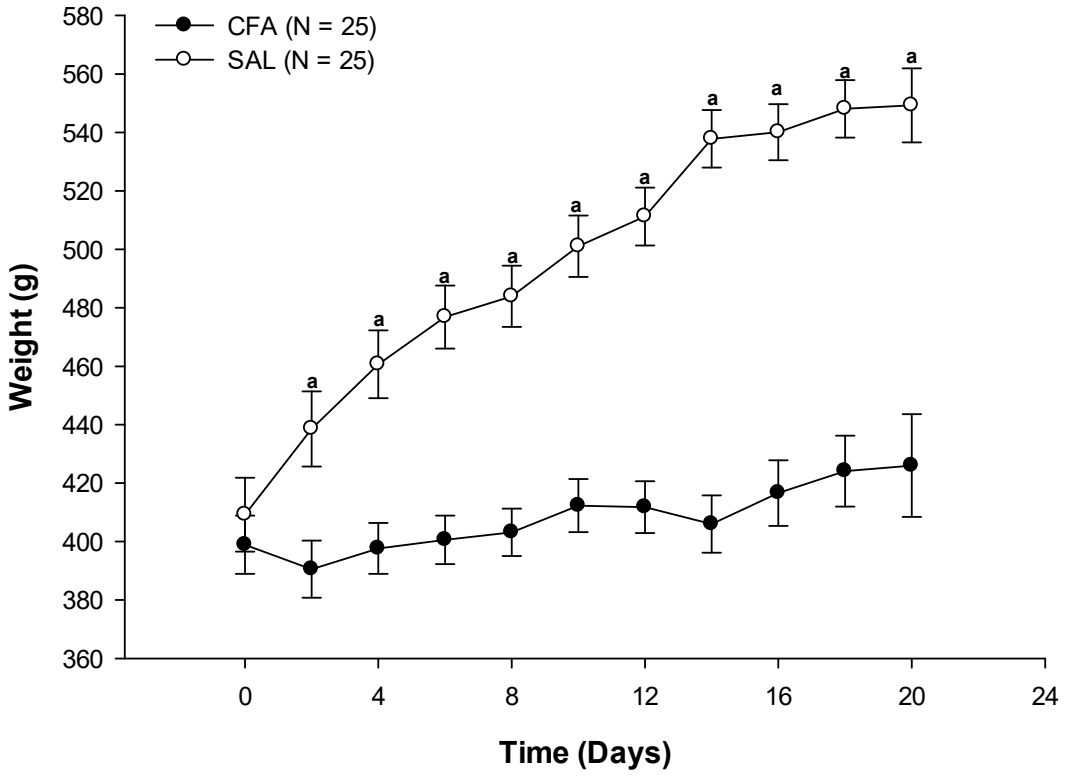


Figure 4. Body weight of animals treated with saline or CFA over time. Mean ± standard error of mean.

[a] significantly different respective value in CFA-treated rats. $p<0.05$.

3.1. Assessment of chronic inflammation

Plasma TNF-alpha

Plasma levels of TNF-α at baseline were not significantly different between CFA- and saline-treated animals. However, the levels of TNF-α in CFA-treated rats became significantly elevated compared to their saline counterparts after 14 days (Figure 5). There was no significant increase in the levels of plasma TNF-α of saline-treated animals.

Paw measurements

Baseline mean ankle and paw volume (left and right) were not significantly different between CFA- and saline-treated animals. The mean displacement volume of the left paws (injection site) of CFA rats became significantly larger compared to the left paw of saline rats at respective measurement points (Figure 6A). Mean displacement volume of contralateral paws became significantly different 14 days after treatment (Figure 6B).

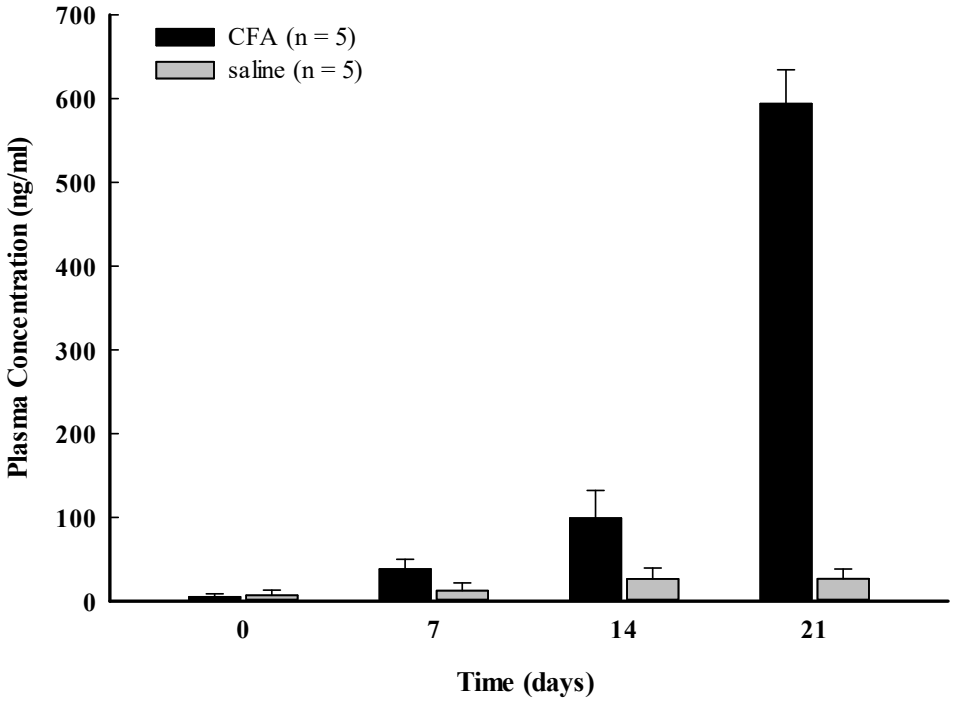


Figure 5. Levels of TNF-α in plasma of inflamed (CFA) and non-inflamed (SAL) animals. Mean ± standard error of mean.

[a] significantly different from saline-treated rats at respective time points. $p < 0.05$.

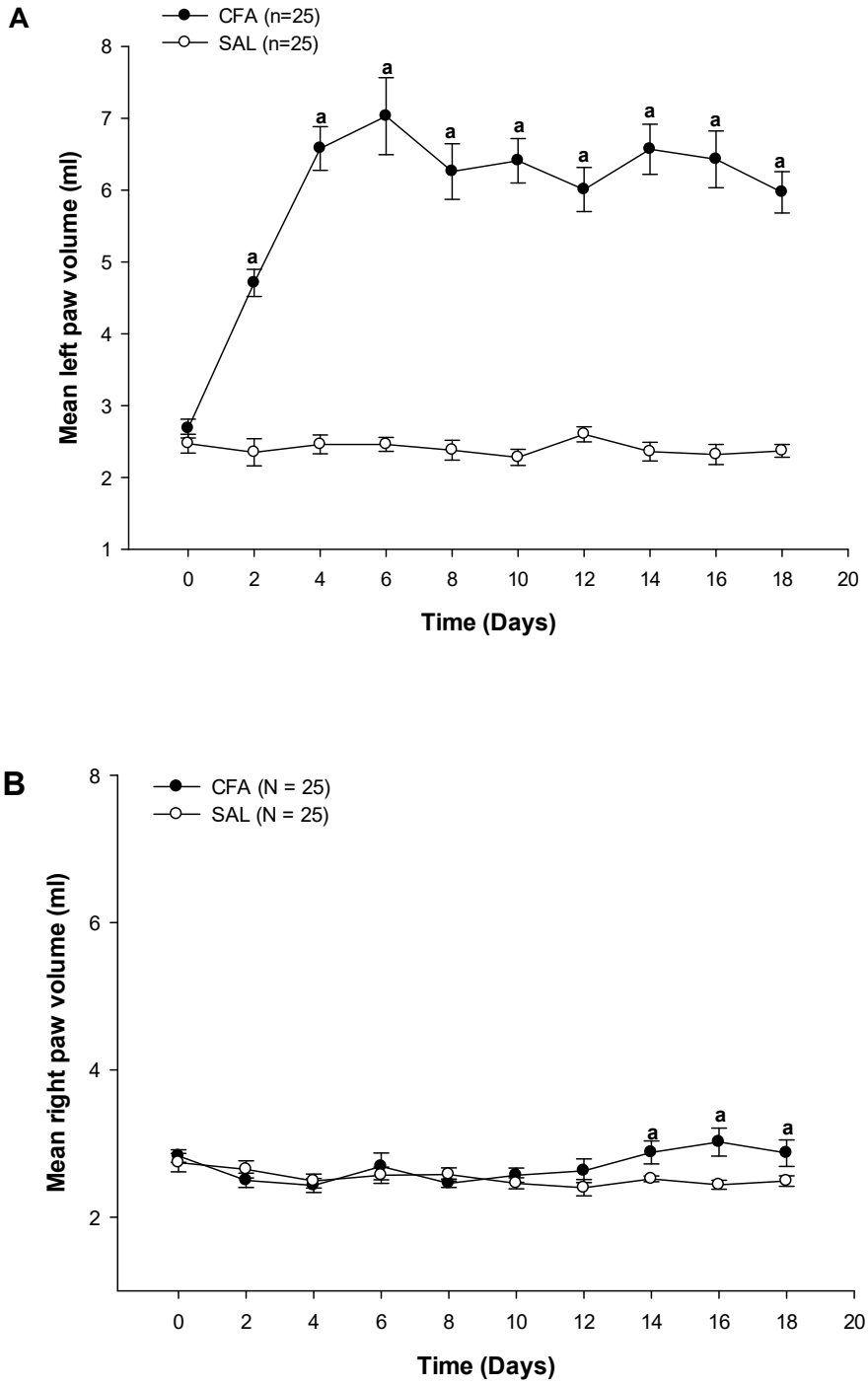


Figure 6. Mean volume of left (A) and right (B) paws of CFA- and saline-treated animals using water displacement method. Mean ± standard error of mean.

[a] significantly different compared to paws of saline-treated rats. $p<0.05$.

3.2. Electric field stimulation responses in rat tail artery

Electrical field stimulation elicited contractions in helical strips of rat tail arteries at 50 V and 0.5 ms pulse width. Progressive increase in frequency of stimulation (1.25 – 40 Hz) resulted in greater contractions. However, EFS-evoked contractions were completely abolished at all frequencies in the presence of tetrodotoxin (TTX; 10^{-7} M), and hence were considered neurogenic in origin.

3.3. Effect of inflammation on EFS-evoked contractions

Electrical frequency stimulations produced significantly greater contractile responses in CFA- compared to saline-treated group of rats at respective frequencies. This difference in response was noted at the low and high frequencies ranging from 2.5 to 40 Hz (Figure 7).

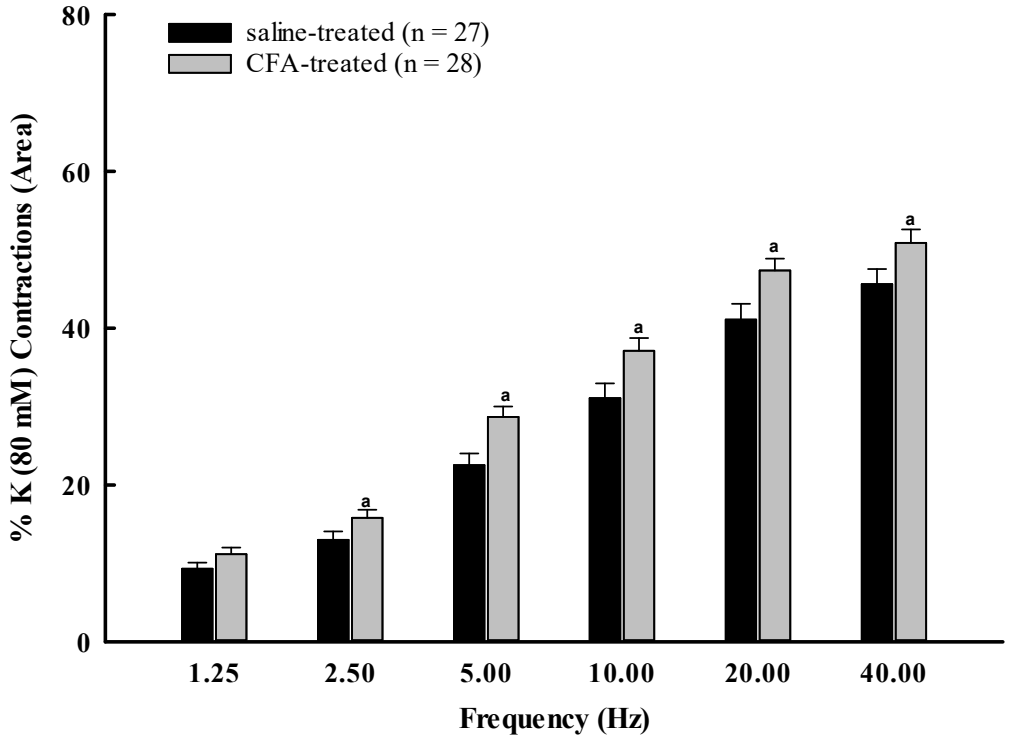


Figure 7. Electric field-evoked contractions in CFA- and saline-treatment groups of rats at frequencies indicated. Mean ± standard error of mean. Contractions were elicited at 50 V and 0.5 ms pulse width for 30 seconds.

[a] significantly different from saline at respective frequencies of stimulation. $P<0.05$.

3.3.1. Effect of Prazosin and WB4101 on EFS-evoked contractions

The presence of the selective α_1 antagonist prazosin (0.3 μM) produced significant reductions in EFS-elicited contractions at higher frequencies of stimulation (10 – 40 Hz) in tail arteries of CFA- and saline-treated rats compared to the respective control responses (Figure 8A & B). In blood vessels of CFA rats, however, prazosin significantly attenuated field stimulated contractions in the frequency range of 1.25 – 5 Hz compared to responses in saline-treated animals (Figure 8B).

Similarly, WB4101 (0.03 μM) significantly attenuated the EFS-evoked contractions in tail arteries of CFA- and saline-treated groups of rats at higher frequencies of stimulation (10 – 40Hz) compared to the corresponding control responses (Figure 9A & B). In blood vessels of CFA rats, however, WB4101 significantly reduced electric field-evoked contractions at lower frequencies of stimulation between 1.25 and 5 Hz, compared to the field-stimulated responses in blood vessels of saline-treated rats. There was no significant difference in the effect of WB4101 on EFS-evoked contractions between CFA- and saline treatment groups.

The pattern of inhibition indicated a greater contribution of α1-adrenoceptors mediated contractions in the blood vessels from CFA-treated animals compared to saline-treated animals (Figure 8A & B; 9A & B).

3.3.2. Effect of rauwolscine on EFS-evoked contractions

There was no significant difference in EFS-evoked contractions in the presence of rauwolscine between CFA- and saline-treated groups of rats, compared to the control responses at respective frequencies of stimulations (Figure 10A & B).

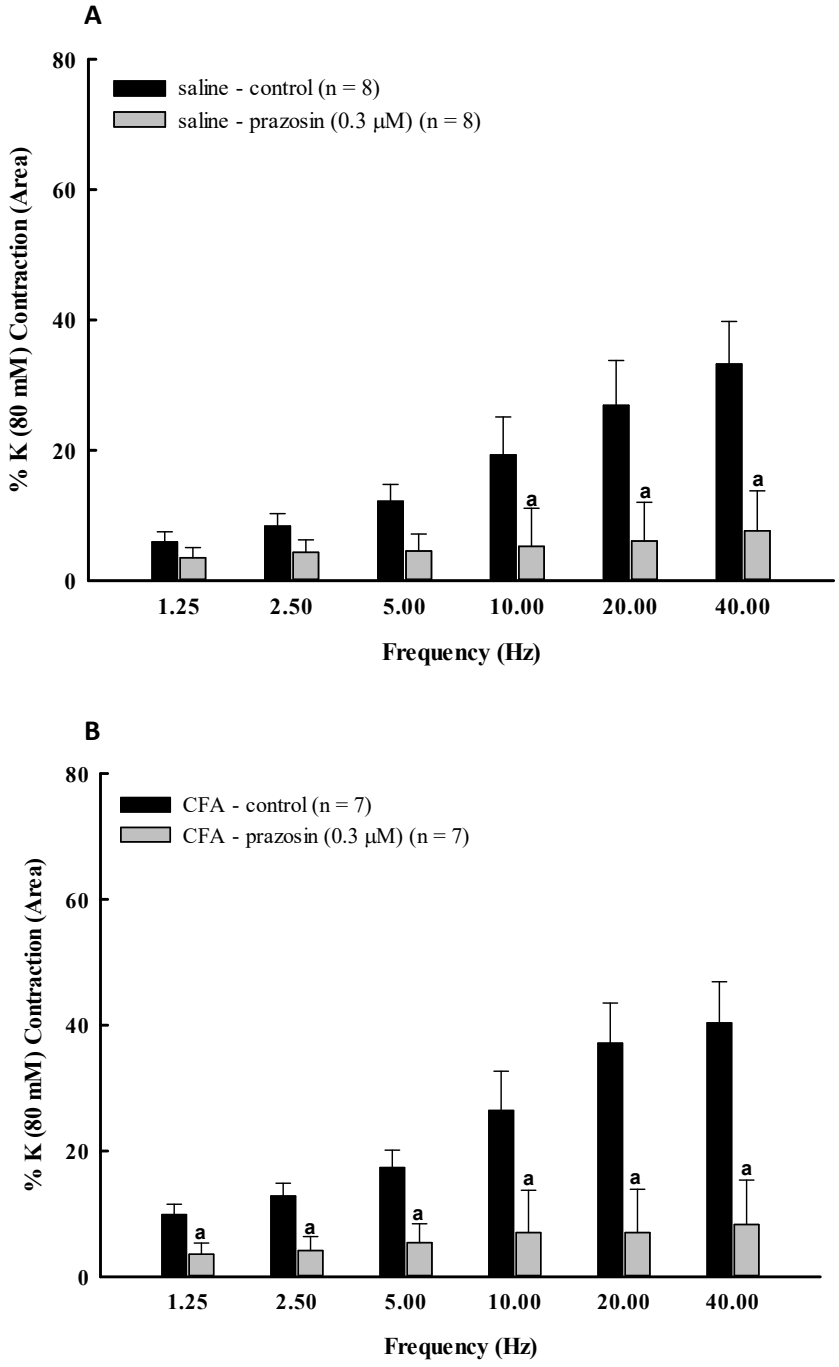


Figure 8. The effect of prazosin on EFS-evoked contractions in tail artery of saline- (A) and CFA-treated (B) rats. Mean ± standard error of mean. Pra, Prazosin. Contractions elicited at 50 V and 0.5 ms pulse width for 30 seconds.

[a] significantly different from control response at frequencies indicated. $p<0.05$.

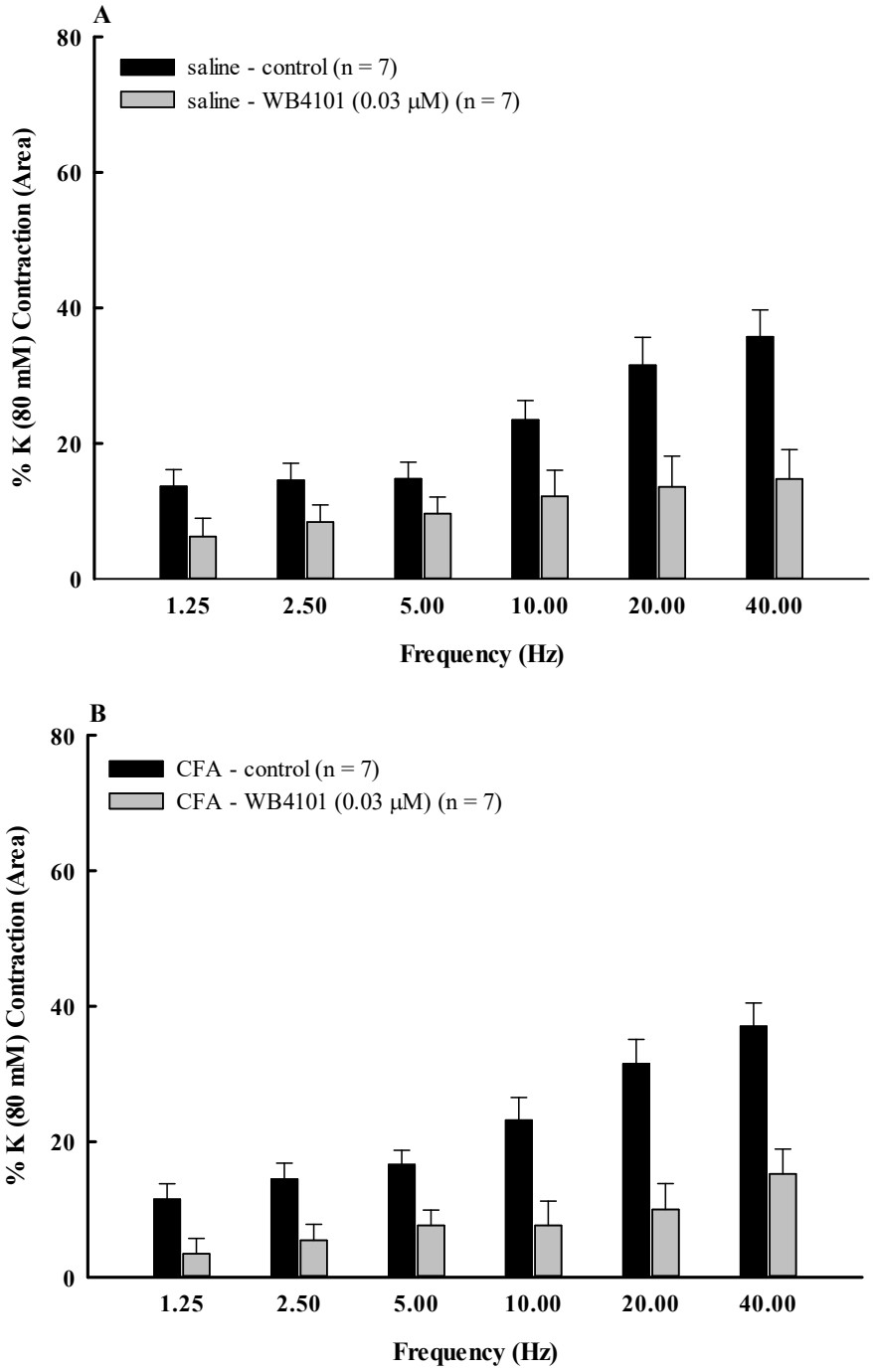


Fig 9. Effect of WB4101 on EFS-evoked contractions in tail arteries of saline- (A) and CFA-treated (B) rats at frequencies indicated. Mean ± standard error of mean. Contractions elicited at 50 V and 0.5 ms width for 30 seconds.

[a] significantly different compared to control responses at respective frequencies. $p<0.05$.

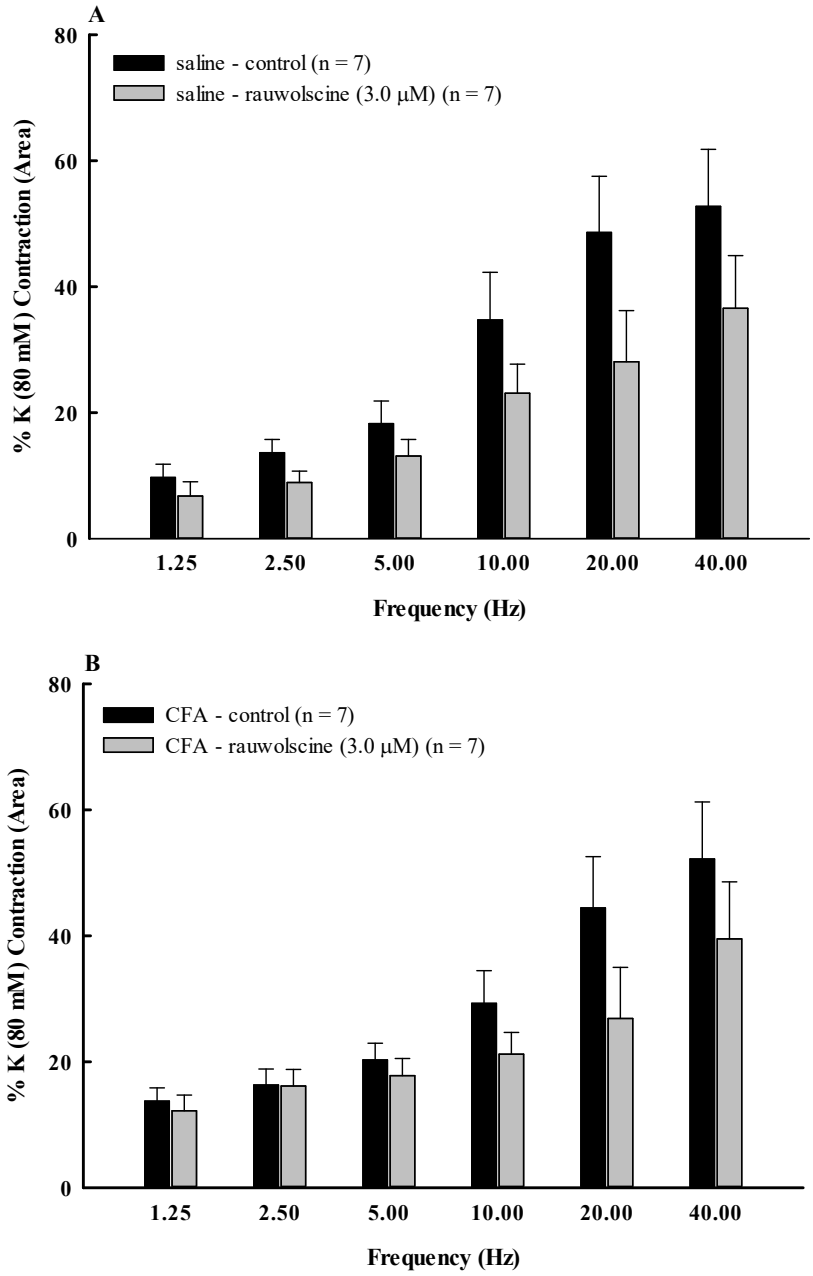


Figure 10. The effect of rauwolscine on the EFS-evoked contractions in saline (A) and CFA (B) treatment groups at frequencies indicated. Mean ± standard error of mean. Contractions elicited at 50 V pulse train and 0.5 ms pulse width for 30s. Rau, Rauwolscine. $p<0.05$.

3.3.3. Effect of cocaine on EFS-evoked contractions

Cocaine significantly enhanced EFS-evoked contractions in tail arteries of CFA- and saline treated animals at frequencies of stimulation between 5 and 40 Hz, compared to the corresponding controls (Figure 11 & 12). There was no significant difference in the effect of cocaine on the EFS-evoked contractions in blood vessels from CFA- and saline-treated group of animals.

Effect of rauwolscine in the presence of cocaine

Rauwolscine (3 μM) had no effect on EFS-evoked contractions in the tail arteries of CFA- and saline-treated rats in the presence of cocaine (1 μM). There was no significant effect of rauwolscine on cocaine-enhanced contractions from blood vessels of CFA and saline-treated groups of animals (Figure 11A & B).

Effect of WB4101 in the presence of cocaine

WB4101 (0.03 μM) significantly attenuated the EFS-evoked contractions in the presence of cocaine (1 μM) compared with the corresponding controls in tail arteries of CFA and saline-treated group of animals between 5 and 40 Hz (Figure 12A & B). In blood vessels of CFA rats, WB4101 also significantly inhibited contractions in the presence of cocaine at the lower frequencies of stimulation (1.25 – 2.5 Hz) (Figure 12B). There was no significant effect of WB4101 in the presence and absence of cocaine between CFA and saline group of animals.

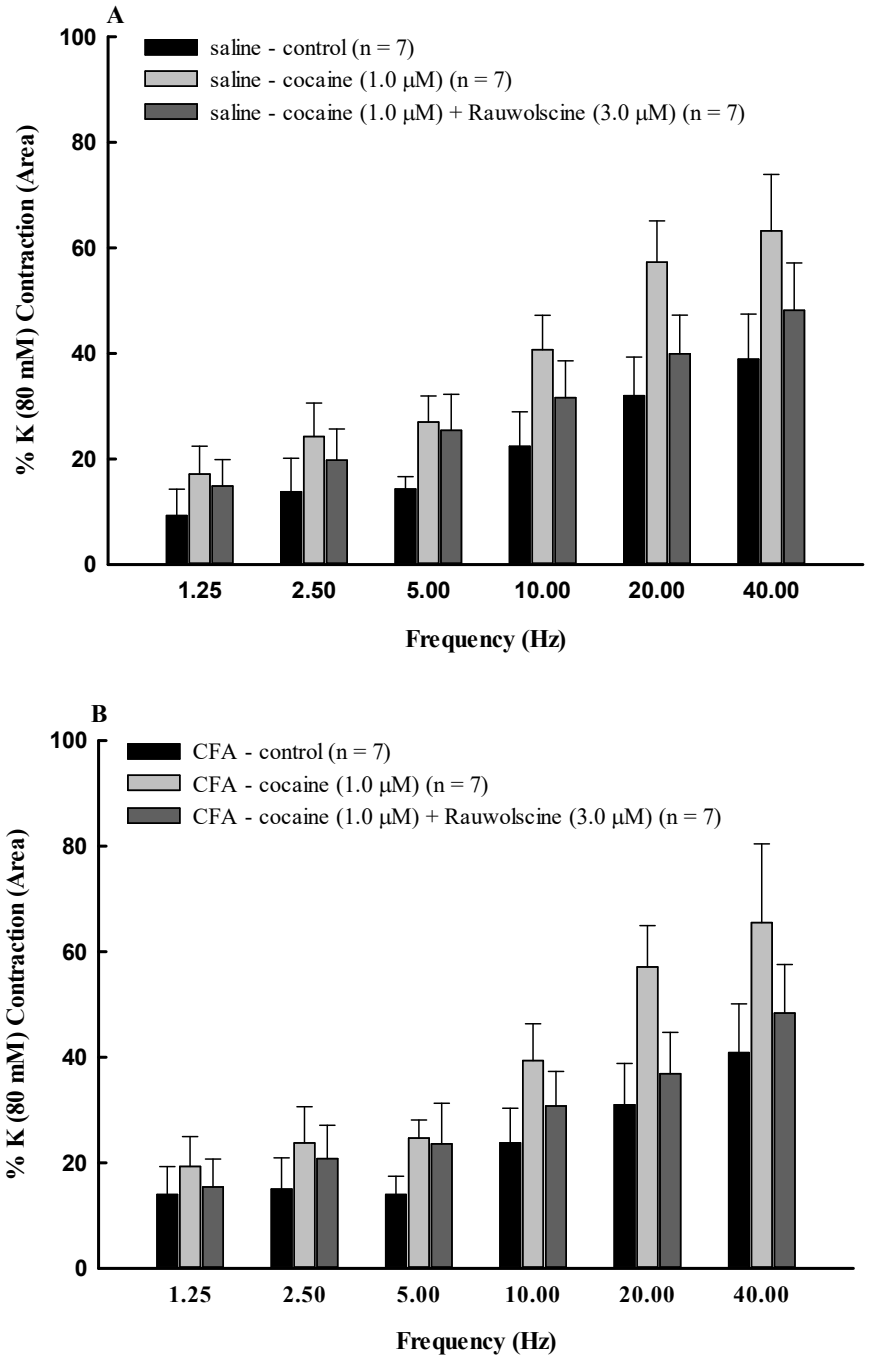


Figure 11. Effect of cocaine in the absence and presence of rauwolscine on electric field-evoked contractions in tail arteries of saline (A) and CFA (B) rats. Contractions elicited at 50 V and 0.5 ms pulse width for 30 seconds. Mean ± standard error of mean. Co, Cocaine; Rau, Rauwolscine.

[a] significantly different from control at respective frequencies. $p<0.05$.

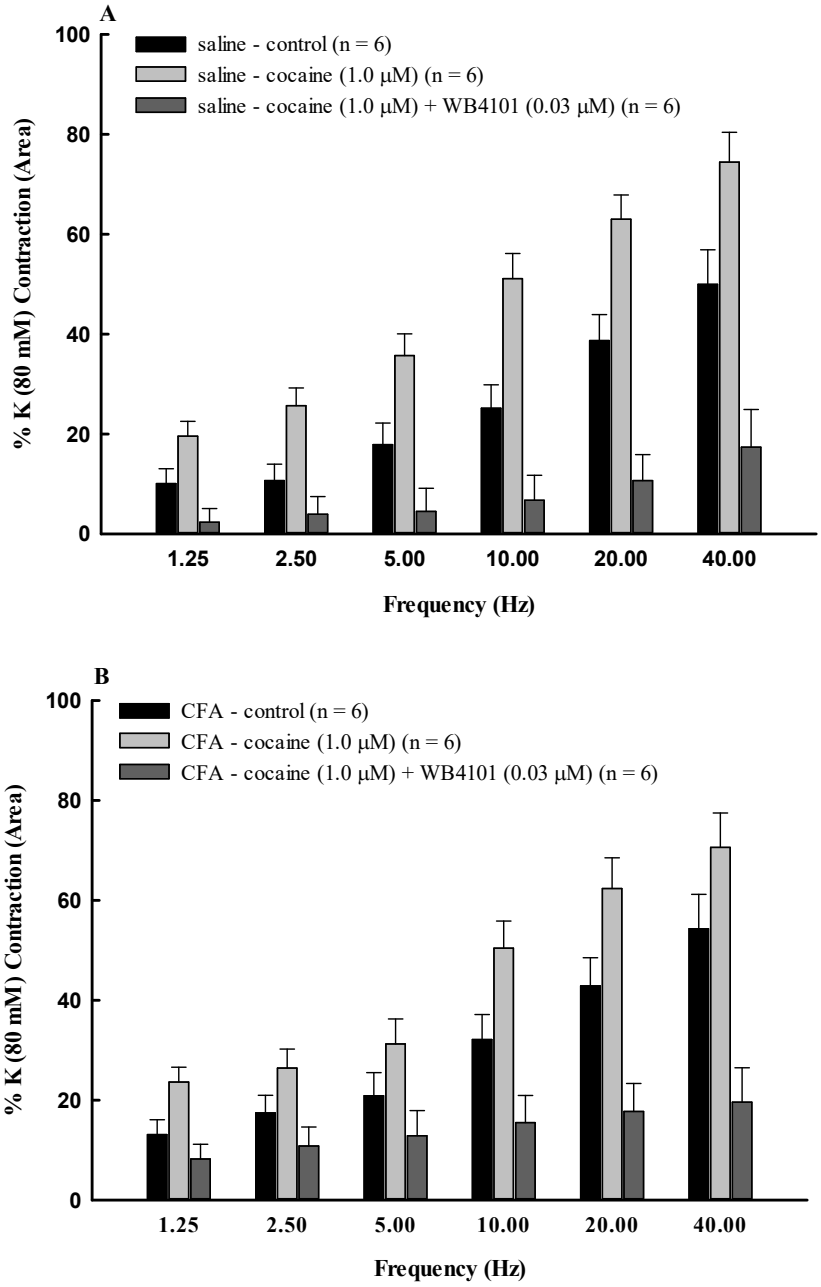


Figure 12. Effect of cocaine on the EFS-evoked contractions in tail arteries of saline (A) and CFA (B) treated animals in the absence and presence of WB4101. Contractions elicited at 50 V and 0.5 ms pulse width for 30 seconds. Mean ± standard error of mean. Co, Cocaine; WB, WB4101.

[a] significantly difference from control at respective frequencies.

[b] significantly different from cocaine-enhanced contractions. $p<0.05$.

3.3.4. Effect of vehicle on EFS-evoked contractions

Water (dH_2O) had no effect on the contractile responses to EFS in blood vessels of both saline and CFA treated rats (Figure 13A & B). Contractile responses were comparable in the cumulative stimulations over time at corresponding frequencies.

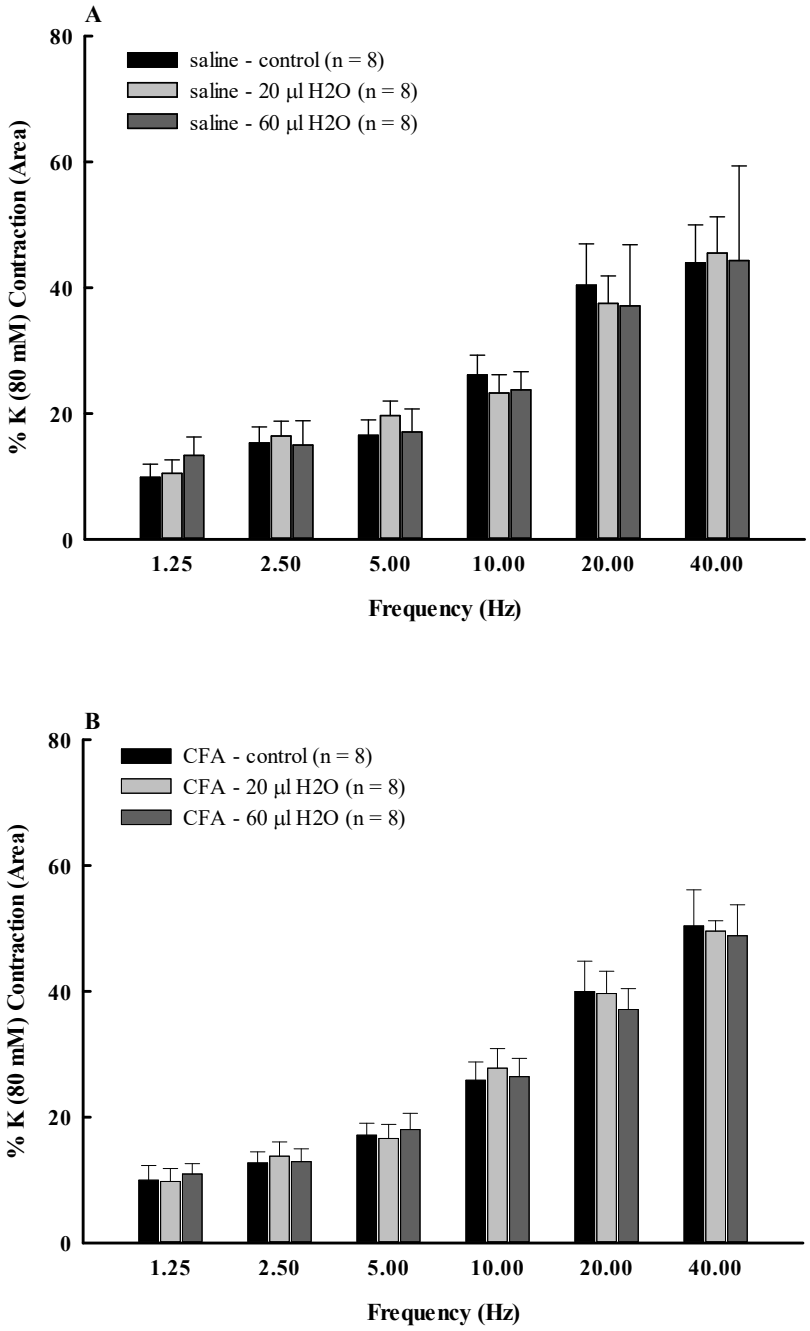


Figure 13. Effect of vehicle on electric field-evoked contractions in tail arteries of saline (a) and CFA (b) treated animals at frequencies indicated. Contractions were elicited at 50 V and 0.5 ms pulse width for 30 seconds. Mean ± standard error of mean.

3.4. Immunofluorescence

Localization of α_1- and α_{2b}- adrenoceptors as well as tyrosine hydroxylase were characterized using immunofluorescent techniques. Immunofluorescence revealed expression of α_1-adrenoceptors on the endothelial cells of blood vessels from CFA-treated but not saline-treated animals. There was similar expression of α1-adrenoceptors on the smooth muscle cells of vascular tissues from CFA versus saline-treated animals (Figure 14).

The presence of α_{2b}-adrenoceptors was more abundant in endothelial cells of blood vessels from saline compared to CFA-treated rats (Figure 15). There was no difference in the enzymatic expression of tyrosine hydroxylase in the vascular tissues from CFA-treated animals compared to saline-treated animals (Figure 16).

Finally, immunofluorescence revealed significant difference in the expression of IBA-1 (DAPI stain) in the adventitia of blood vessels from CFA-treated animals compared to blood vessels from non-inflamed animals (Figure 17). These data suggest massive infiltration of the sympathetic nerve plexus with macrophages in the adventitia.

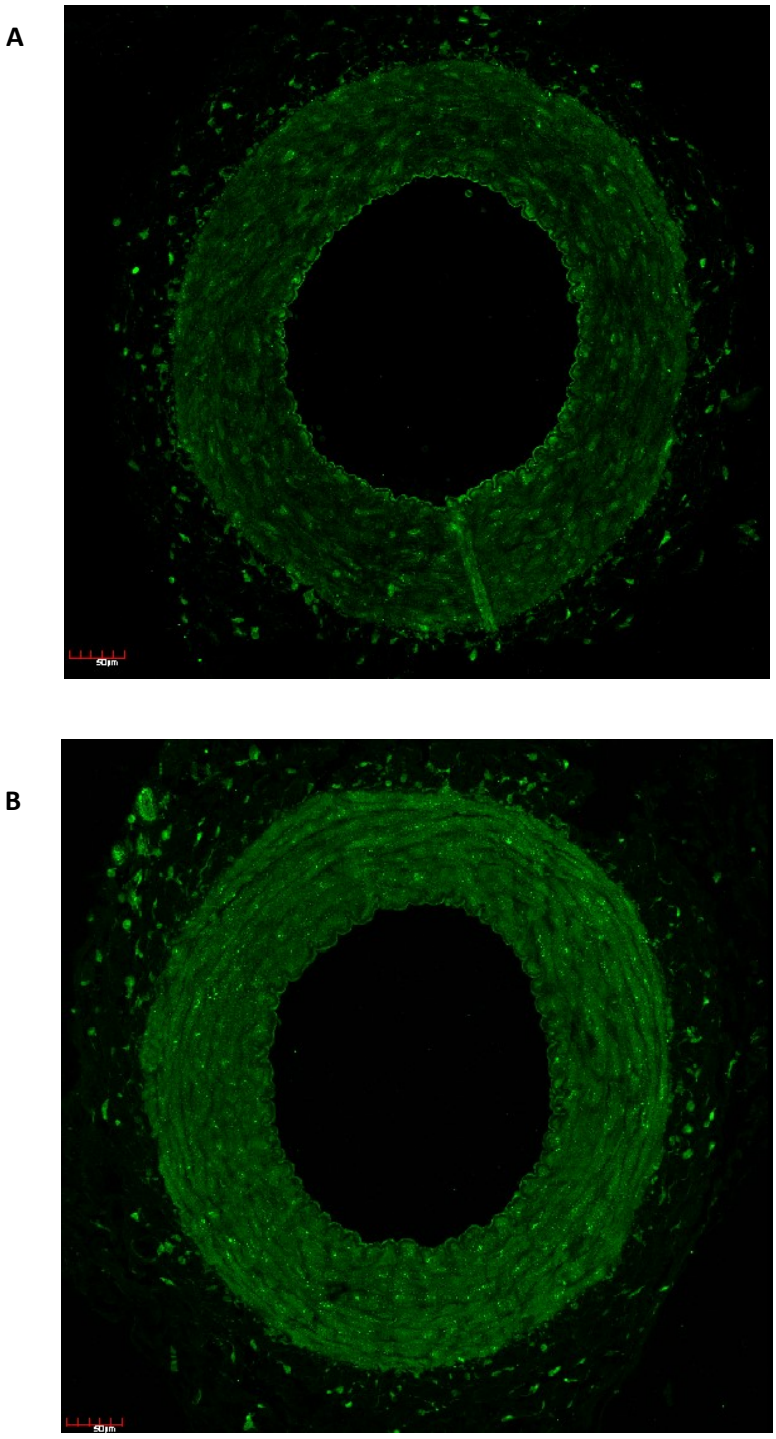


Figure 14. Qualitative expression of alpha$_1$-adrenoceptors in tail arteries of CFA-treated (A) and control (B) rats. CFA, experimental rats; SAL, control rats.

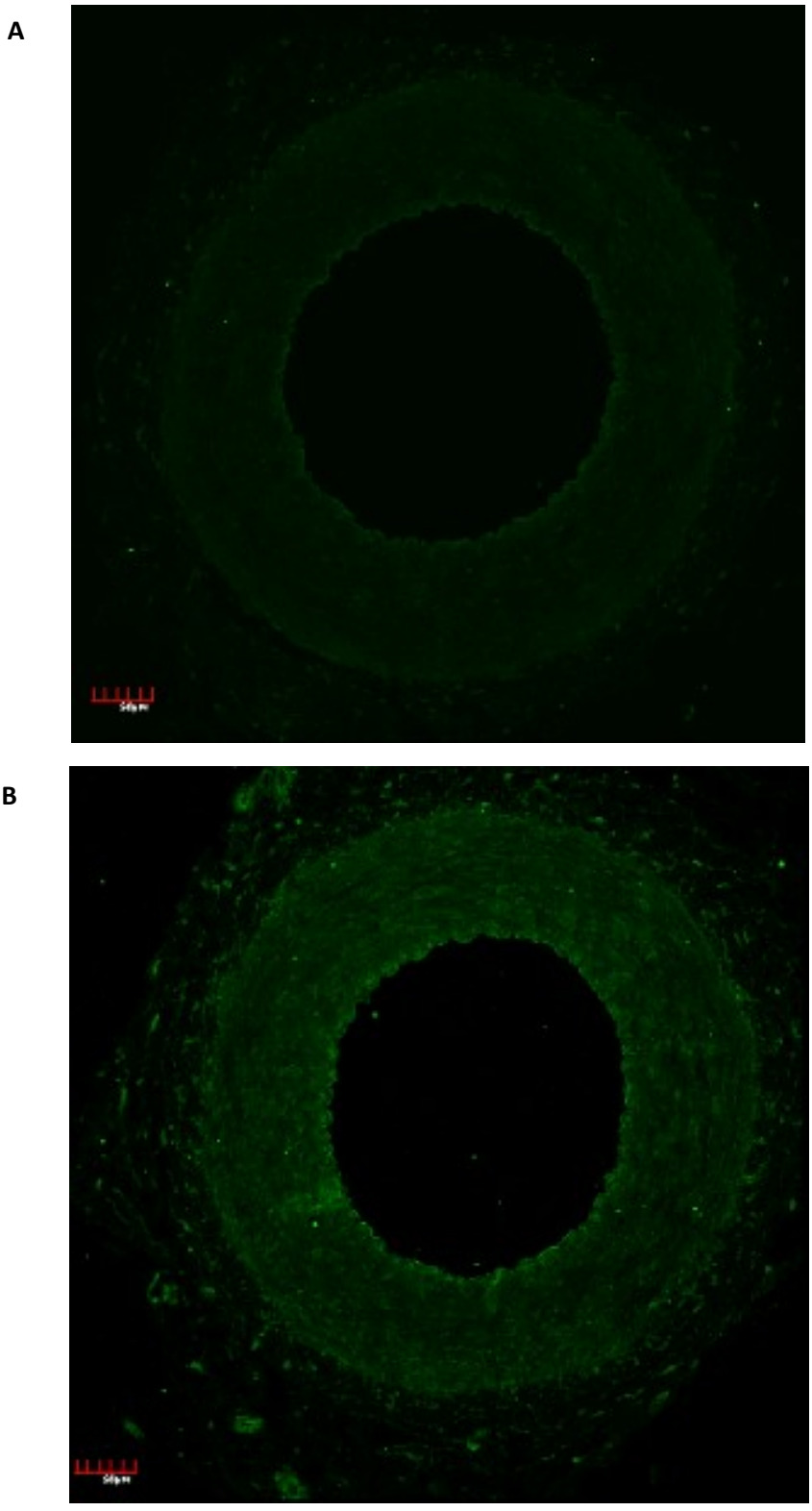


Figure 15. IHC expression of alpha2_b in blood vessels of CFA-treated (A) and control (B) animals.

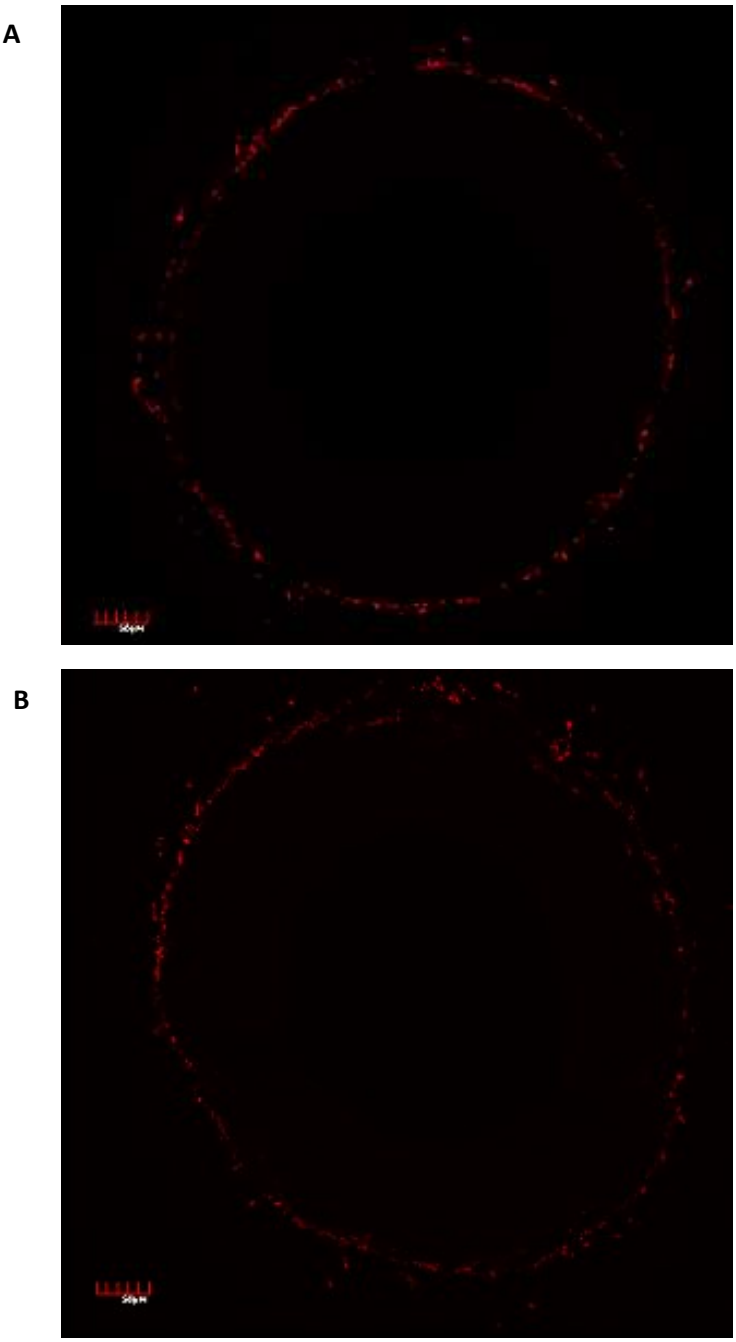
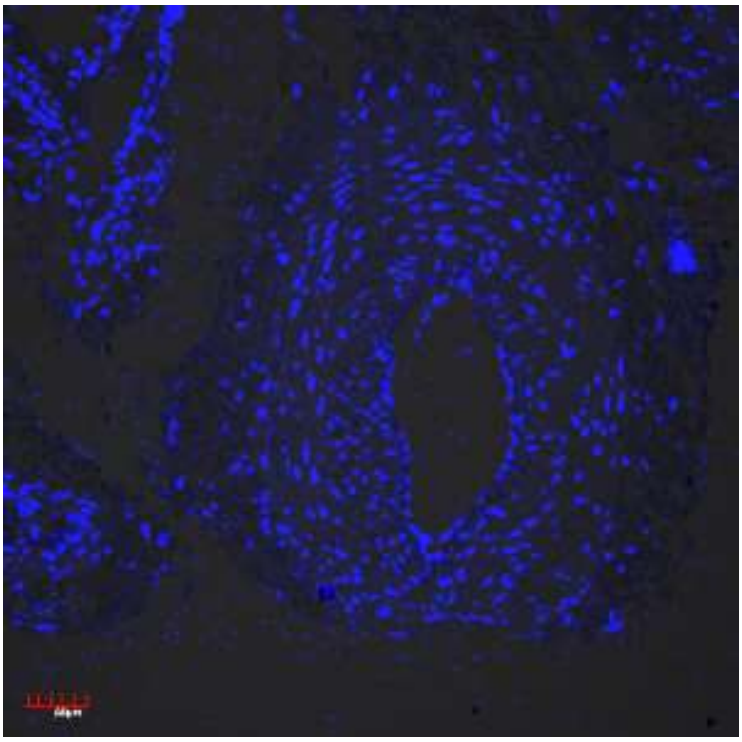


Figure 16. Immunofluorescent expression of tyrosine hydroxylase in blood vessels of CFA-treated (A) and saline-treated (b) rats.

A



B

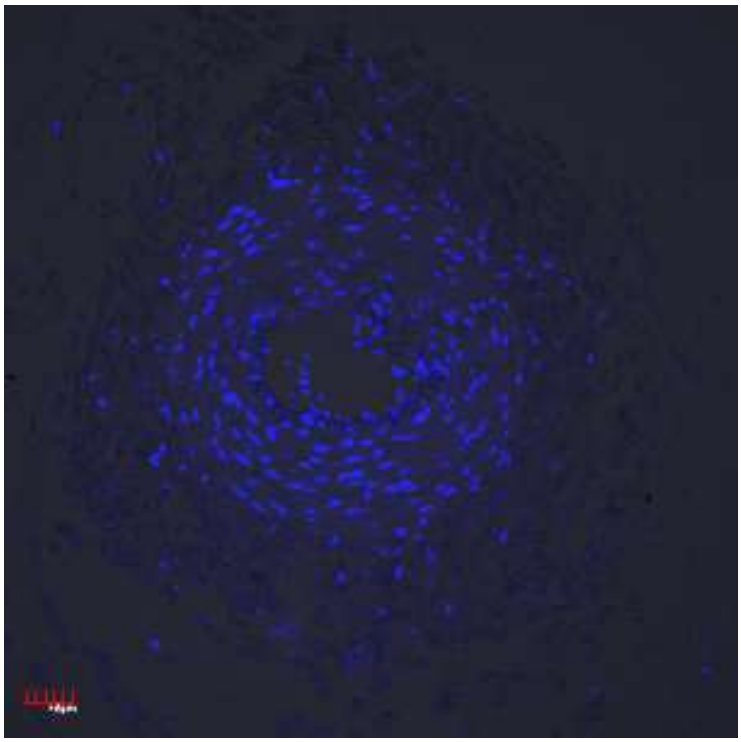


Figure 17. Examples of DAPI stain of IBA-1 expression in blood vessels from CFA-treated (A) and control (B) animals.

4.0. Discussion

In the present study, the effect of chronic inflammation on field stimulation evoked contractions in rat tail arteries was investigated. Induction of inflammation resulted in significant elevation in the levels of plasma TNF-α in CFA-treated animals compared to the saline-treated animals. Electrical field stimulation-evoked contractions in arteries of CFA animals were significantly greater than in saline animals. The selective sodium channel blocker, tetrodotoxin abolished the field stimulation-evoked contraction, confirming their neurogenic nature.

Contractions elicited at lower frequencies were significantly attenuated by alpha$_1$-adrenergic antagonism in arteries of CFA-treated rats compared to saline-treated animals. IHC expression of alpha$_1$-adrenergic receptor observed in the endothelium of blood vessels of inflammed rats differed from α_1 expression in blood vessels of control animals. Similarly, IHC revealed differential expression of alpha$_{2b}$-receptors in the endothelium of blood vessels of CFA-treated versus control rats. The effect of the neuronal uptake inhibitor cocaine, in enhancing electrical evoked contractions were comparable in blood vessels of CFA-treated and saline-treated animals. Furthermore, evidence seem to indicate the neurogenic response mediated by α_2-adrenoceptors did not play a significant role in the process of contraction, since neurogenic contractions were also not affected by alpha$_2$-receptor antagonism in inflammed and non-inflammed animals.

4.1. Tumor Necrosis Factor - alpha

Inflammation is a normal biological response to foreign particles. However, continuous exposure to a foreign stimulus causes chronic inflammation (Warrington et al., 2011). Sprague-Dawley rats represent an excellent model for chronic inflammation *in vivo* (Schopf et al., 2006). Serum levels of cytokines TNF-α and IL-6 have been reported to be consistently elevated after CFA injection with peak plasma levels between days 12 and 19 (Schopf et al., 2002).

In this model of adjuvant-induced arthritis, previous investigations have found an elevation in the levels of cytokines in systemic circulation as well as increased mean volume of the injected and counter-lateral paws of animals (Weichman, 1989). Similarly, Smith-Oliver et al. (1993) have also detected elevated levels of TNF-α and IL-6 in serum of adjuvant arthritic rats. In line with these findings, Phillipe et al. (1997) also noted a spike in serum TNF-α and IL-6 12 hours after CFA injection in adjuvant arthritis rats. In addition, it has also been reported that significant increases in plasma TNF-α levels in adjuvant-induced arthritic rats occur between 11 and 25 days after CFA injection (Szekanecz et al., 2000).

In present study, CFA caused inflammation with a significant increase in plasma levels of TNF-α and mean paw volume in left and right hind paws of CFA-treated rats over time. These findings are in accord with previous evidence in the literature, and that seem to have a systemic component (Schopf et al., 2006). The systemic manifestation of the response is evident by the elevated levels of cytokines in peripheral circulation and swelling of the contralateral paws.

4.2. Effects of inflammation on sympathetic nerve response in tail artery

Alpha-adrenoceptor activation mediates the effects of sympathetic nerve stimulation in vascular tissue. The rat tail artery is densely innervated with sympathetic nerves that release noradrenaline. Electrical field stimulation of sympathetic nerves causes the release of neurotransmitter from neurons which then activates α-adrenoceptors leading to vasoconstriction (Stjärne, 2000). In the current study, neurogenic contraction as calculated by the area under curve was greater in blood vessels from inflamed rats compared to non-inflamed rats. Based on this observation, it is possible that the neuroeffector pathways leading to vascular smooth muscle function in chronic inflammation may have been altered.

A viable explanation could be that the elevated expression of plasma TNF-α may have contributed to the enhanced activation of adrenergic receptors mediated by sympathetic neurotransmission. Thus, the pro-inflammatory effect on sympathetic nerves could have contributed to (i) the increased release of the transmitter noradrenaline from sympathetic nerve endings, or (ii) enhanced response at the receptor level, or both. There is evidence that elevated levels of TNF-α in acute septic conditions upregulate sympathetic nerve activity at the neuronal level (Xie et al., 1993) through increased intracellular Ca^{2+} signaling (Shu et al., 2007; Dellinger et al., 2008; Lukewich et al., 2014). On this premise, one could postulate that an increased release of noradrenaline, hence increased adrenergic receptor activation, leads to the observed increase in vascular smooth muscle contraction. However, the effect of the neuronal uptake inhibitor, cocaine, in our current data does not support this possibility, suggesting perhaps an alternative explanation. All the same, more studies are needed to

investigate further the role of inflammatory cytokines (in chronic inflammation) on the neurogenic release of noradrenaline.

It is also possible that TNF-α may have affected adrenoceptor activation by altering the signaling pathway processes responsible for vascular contraction. On this premise, alterations in the phospholipase C signaling pathways could have led to changes in the contractile response in blood vessels of inflamed rats. In addition, because vasoconstrictor-stimulated Inositol 1,4,5-triphosphate (IP_3) formation is also dependent on extracellular Ca^{2+} (Brock et al., 1989; Morimoto et al., 1990, Dickenson et al., 1993), the indirect activation of PLC in smooth muscle by circulating TNF-α could have contributed in some way to the current observation (Garland and Angus, 1996). Comparatively, there is evidence that the inflammatory response is modulated by a cross-talk between the immune system and the central nervous system. This dual regulation could involve hormonal and neuronal mechanisms through the hypothalamic-pituitary-adrenal (HPA) axis and may impact the release of noradrenaline from the sympathetic nerves. Here, the sympathetic nervous system provides feedback to organs regulating the inflammatory response through the secretion of catecholamines from postganglionic sympathetic neurons (Lukewich et al., 2014). In turn, the immune system may also affect the CNS through the release of cytokines (Eskandari et al., 2003). In addition, TNF-α and IL-6 have been found to induce changes in vascular physiology *in vivo* (Tracey and Cerami, 1994; Holden and Pakula, 1996). Sternberg (1997) has shown that TNF-α can stimulate neuroendocrine responses in the CNS of animals *in vivo* through activation of the hypothalamic-pituitary-adrenal (HPA) axis. On the basis of this regulatory interplay, further investigation might provide insight into the effects of cytokines and chronic inflammation on the sympathetic nerve-

mediated vascular contractions, *in vivo*. Therefore, the elevation of plasma TNF-α levels and other cytokines during the inflammatory process may have caused permanent change in the alpha adrenoceptor-mediated effects. This may have resulted in the modest, but significant, enhancement of neurogenic contraction observed in the tail artery of inflamed animals.

Alpha-adrenoceptor blockers invariably inhibit the effects of sympathetic nerve-released noradrenaline by competitively blocking the activation sites of post-junctional receptors. Ligand-binding studies have characterized alpha-adrenoceptor subtypes by their effect on sympathetic nerve activation. Zacharia et al. (2004) have demonstrated that α_1-adrenergic receptors play a predominant role in blood vessel contractile responses to sympathetic nerve activation compared to α_2-adrenergic receptors. Kimikihara et al. (2007) have also demonstrated that specific α_1-subtypes play a role in smooth muscle contractions in proximal segments of rat tail arteries. Furthermore, as demonstrated by Zacharia et al. (2013), α_{1A}- and α_{1D}-adrenoceptor subtypes contribute to over fifty percent of contractile response to sympathetic activation in vascular tissues. The selective α_1 antagonist prazosin has been shown to exhibit selective antagonism to α_1-adrenoceptor activation (Bao and Stjärne, 1993; Zacharia et al., 2004; Zacharia et al., 2013). In addition, the α-adrenoceptor antagonist WB4101 is known to significantly inhibit nerve-mediated contractions in vascular smooth muscles, whereas the selective α_2 blocker rauwolscine has been found to only modestly inhibit the neurogenic contractions in vascular tissues of rats and pigs (Bao and Stjärne, 1993; Duggan et al., 2011).

In the present study, prazosin and WB4101 had a variable effect on field stimulation-evoked contractions in tail arteries of CFA-treated rats compared to saline-treated rats. Prazosin had a significant effect on vasoconstriction at higher frequencies of stimulation in both

inflamed and non-inflamed rats, but the antagonism was also evident at lower frequencies of stimulation in inflamed animals. Possible explanations for this effect are (i) an alteration in the sensitivity of the adrenoceptors and/or receptor affinity for the selective ligands or (ii) an alteration in the post-receptor mediated events, i.e., the signaling processes leading to excitation-contraction coupling. Prazosin has been found to inhibit contractions due to electrical-field stimulation in rat femoral and pulmonary arteries in the frequency range of 5 – 30 Hz (Zacharia et al. 2004; Duggan et al., 2011). However, the effective blocking at the lower frequencies (1.25 – 2.5 Hz) in vascular smooth muscle of CFA-treated rats could also suggest that α_1-adrenoceptor in tail arteries from CFA rats were more sensitive to α_1 blockade relative to controls. Furthermore, characterization of transmission from peripheral sympathetic nerves to vascular smooth muscles has demonstrated that the largest smooth muscle responses occur in the low frequency range between 0.2 – 0.5 Hz in conscious rats (Stauss and Kregel, 1996; Stauss et al., 1997). This physiological frequency range of stimulation is also believed to be the most important in the sympathetic modulation of vasomotor activity in rats and humans (Milliani et al., 1991; Stauss et al., 1998). In addition, Luckensmeyer and Keast (1998) demonstrated a similar finding in smooth, intestinal muscles in a 0.5-5 Hz frequency range of stimulation. These observations emphasize the physiological relevance of alterations in α_1-adrenoceptor activation as noted here in the low frequency range of CFA-treated rats.

Conversely, this study also found no effect of α_2 blockade on the neurogenic contractions in blood vessels of inflamed and non-inflamed animals. This corroborates previous findings that α_2-adrenoceptors play only a modest role in mediating vascular smooth muscle contractions (Bao et al., 1993; Bao and Stjärne, 1993; Zacharia et al., 2004). Furthermore, there

was no effect of inflammation in the physiological response to α_2-adrenoceptor activation, suggesting that the difference in EFS-evoked contractions in blood vessels from inflamed and non-inflamed rats were unlikely due to alteration in α_2-adrenoceptor-mediated responses. Nevertheless, there is evidence that inflammation impacts the negative feedback functioning of pre-synaptic α_{2A}-adrenoceptors in vivo, leading to increased release of noradrenaline (Hoch et al., 2015). It is still unclear, however, whether the α_2-adrenoceptors involved were adrenergic i.e. activated by noradrenaline or some other endogenous neurotransmitter. Hence, this pattern of inhibition indicated a greater contribution of α_1-adrenoceptors (versus α_2-adrenoceptors) in sympathetic-mediated contractions in blood vessels of animals with chronic inflammation compared to controls.

The small residual contractions in the presence of WB4101 and prazosin could be indicative of the action of other neurotransmitters released from sympathetic nerves other than noradrenaline, e.g. adenosine triphosphate and neuropeptide Y. Purinergic receptors have been identified in vascular smooth muscle cells and play an important role in physiological responses (Harhun et al., 2015). Interestingly, electrical field-stimulation also evoke the release of adenosine triphosphate (ATP) from sympathetic nerve terminals (Bao and Stjarne, 1993; Bao et al., 1993). Hence, the observed contractions are presumably the result of the dual contractile effects of NA and ATP through the activation of adrenergic and purinergic receptors, respectively. However, Bao and Stjarne (1993) demonstrated that P_2x receptor activation only contributes to the initial rapid phase in neurogenic contraction. Still, the possible effect of inflammation on ATP-mediated purinergic activation remains unknown.

In the present study, cocaine significantly enhanced electric field evoked contractions in the frequency stimulation range between 5 and 20 Hz. The potentiating action of cocaine on sympathetic nerve-mediated contractions has been attributed to the inhibition of neurotransmitter reuptake (Bao et al., 1993; Duggan et al., 2011). By inhibiting the reuptake of noradrenaline from the "synaptic cleft", cocaine causes a prolonged effect of noradrenaline on the post-synaptic alpha-adrenoceptors and enhances nerve-mediated contractions. Duggan et al. (2011) demonstrated potentiation of responses in rat pulmonary arteries to cocaine, as did Docherty (2014), who showed an increase in noradrenaline potency in rat vas deferens stimulated to exogenous noradrenaline in the presence of cocaine. This finding suggests a prolonged activation of the post-synaptic receptors or the increased potency of receptors to noradrenaline. Previous studies have reported cocaine-enhanced responses at low (0.1 – 1.0 Hz) but not high (2.0 – 16 Hz) frequency stimulations (Webb et al., 1980). This is at variance with the present findings and those of Stjärne et al. (1994) and Zacharia et al. (2004). However, in the current study, there were no apparent differences in the mechanisms of neuronal uptake in tail arteries from CFA-treated vs saline-treated rats which suggests that the observed differences in EFS-evoked contractions are not linked to the uptake of the noradrenergic transmitter.

4.3. Immunofluorescence

Alpha$_1$ receptor expression in the tail vasculature of CFA-treated animals was different compared to saline-treated rats as characterized by immunofluorescence. In vascular tissue, α_1 adrenoceptors are predominantly found in smooth muscle cells (Skinner et al., 1994; Bylund et al., 1998; Garland et al., 2004; Zacharia et al., 2004; Tripathi et al., 2015). It is also known that

sympathetic activation of these α_1-adrenoceptors mediates smooth muscle contraction in blood vessels. In the current study, α_1 receptors were observed in vascular smooth muscle cells of CFA- and saline-treated rats. In the vascular endothelium of CFA-treated rats, expression of $alpha_1$ receptors deviated from $alpha_1$ characterization in previous studies of receptor localization in blood vessels. This unique expression of $alpha_1$ in the endothelium could represent a difference in adrenoceptor expression in the caudal vasculature of inflamed rats. It is possible that the α_1-adrenoceptor activation in the endothelium may have led to the activation of different intracellular signaling pathways or represent a modified role of endothelial cells in the excitation-contraction coupling mechanism of blood vessels. Release of vasodilatory agents from the endothelium of blood vessels mediates smooth muscle relaxation and vasodilation (Félétou and Vanhoutte, 2000; Vanhoutte, 2009). Previous studies suggest that sympathetic activation of adrenoceptors inhibits the release of nitric oxide from the vascular endothelium (Liao and Homey, 1993; Hijmering et al., 2005). Furthermore, Kamper et al. (2005) found that sympathetic activation of peripheral α_1-adrenoceptors attenuates the nitric oxide-mediated vasodilation, suggesting an inhibition of NO by an α-adrenergic mechanism. It is therefore conceivable that the activity of α_1-adrenoceptors in the vascular endothelium of blood vessels from CFA-treated animals could have contributed to greater contractions through inhibition of the vasodilatory mechanisms.

In intact tail arteries, immunofluorescence showed reduced expression of $alpha_{2b}$-receptors in the endothelium of blood vessels from CFA-treated rats compared to blood vessels from saline-treated rats. $Alpha_{2b}$-adrenergic receptors are predominantly located in the endothelium of vascular tissues but not in the smooth muscle layer of rat tail arteries (McNeill

et al., 1999). Activation of post-junctional α_2-adrenoceptors by exogenously applied noradrenaline mediate modest contraction and endothelium-dependent relaxation (Flavahan et al., 1984; Majmudar et al., 1999) of the vascular smooth muscle cells. A recent study by Thang et al. (2015) found that TNF-α and macrophage infiltration in vascular adventitia inhibits α_2-AR function causing increased release of noradrenaline from sympathetic nerves. Laukova et al. (2010) showed a decrease in gene expression of α_2-adrenergic receptors in stress situations, assessed by the expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-18) in the spleen from rats. It is possible that TNF-α may have down-regulated the expression of α_2 receptors in a similar fashion. The functional relevance of this difference in α_2-adrenoceptors in the endothelium could reflect a disturbed vasodilatory response of α_2-receptors activation in blood vessels from inflamed rats. However, this immunochemical difference in α_{2b}-adrenoceptor expression was not reflected in our functional studies since α_2-adrenoceptor antagonism had no effect on the electrical field-evoked contractions of blood vessels.

4.4. Limitations to study

The present investigation did not quantify sympathetic noradrenaline release to compare SNS activation in blood vessels of inflamed versus non-inflamed animals. It also did not investigate the effect of a joint α_1- and α_2-adrenoceptor blockade on nerve-mediated contractions in the tail artery, whose joint action may further contribute to an altered signaling or contraction mechanism in blood vessels of inflamed or non-inflamed animals.

The administration of analgesic (buprenorphine) for pain management in the CFA-treated animals could also present a limitation to the current study.

Furthermore, in the present study, the effect of inflammation on nerve-mediated vasoconstriction was examined in rat tail arteries *in vitro*. *In vivo*, the interplay between the immune system and CNS also determines how the inflammatory response influences the sympathetic nervous system and vascular function. Hence, our current data may underestimate the contribution of cytokines and the interplay between the inflammatory response and the sympathetic nerves could potentially have on the nerve-mediated physiological effects under *in vivo* conditions, as the effects of cytokines are short-lived. The question of whether the *in vitro* study directly translates in the in vivo state is unclear.

4.5. Future directions

The present study supported the hypothesis that various components of the neurogenic mediated vasoconstriction and vascular function are altered in tail arteries of inflamed animals. The findings also present evidence to suggest that the observed changes in vascular function are mediated chiefly by α_1-adrenoceptor activation and distribution in animals attained by chronic inflammation. The findings of the present study are important in understanding the pathophysiology of vascular diseases mediated by chronic inflammation, for example, atherosclerosis.

It would be interesting to test whether the simultaneous effects of prazosin and rauwolscine in the vascular smooth muscle function of inflamed and non-inflamed rats compare to the effects of WB4101. In addition, to compare the effects of prazosin on EFS-induced contractions in blood vessels of inflamed and non-inflamed rats in the presence of cocaine.

There is speculation that increased systemic levels of TNF-α could induce smooth muscle contractions through sympathetic activation of adrenoceptors or other transduction pathways that contribute to vascular smooth muscle contractions. This concept is supported by the presence of adrenoceptors on immune system organs and the bidirectional relationship between the immune system and the central nervous system in modulating the inflammatory response. Hence, future studies investigating the direct effect of exogenously applied cytokines (e.g. TNF-α or IL-6) on the neurogenic contractions in tail arteries of rats would provide more understanding on the contribution of circulating cytokines *in vivo* on sympathetic nerve and vascular function in persons afflicted with chronic inflammation.

5.0. References

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6.0. Appendix

Table AI: Pharmacological treatment groups for electric field-evoked contraction studies of tail arteries from CFA-treated and saline-treated animals.

Frequency Response Curve Drug	Drug 1 (M)	Drug 2 (M)
Deionized water (Vehicle)	20 µl	60 µl
Prazosin	Prazosin: 10^{-7}	Prazosin: $3x10^{-7}$
Rauwolscine	Rauwolscine: 10^{-6}	Rauwolscine: $3x10^{-6}$
WB4101	WB4101: 10^{-8}	WB4101: $3x10^{-8}$
Cocaine + Deionized water	Cocaine: 10^{-6}	Deionized water: 60 µl
Cocaine + Rauwolscine	Cocaine: 10^{-6}	Rauwolscine: $3x10^{-6}$
Cocaine + WB4101	Cocaine: 10^{-6}	WB4101: $3x10^{-8}$

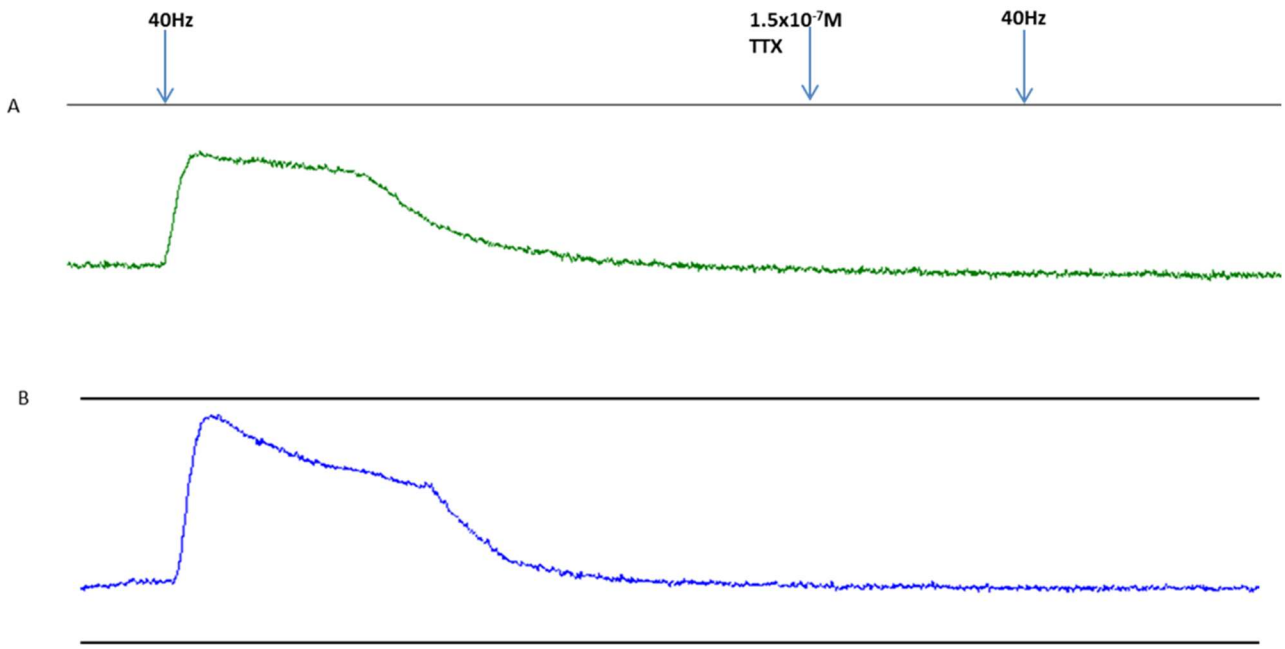

Figure AI: Effect of tetrodotoxin on field stimulation-evoked contractions at 50 V and 0.5 ms pulse in tail arteries of saline-treated (A) and CFA-treated (B) rats at the frequency indicated.

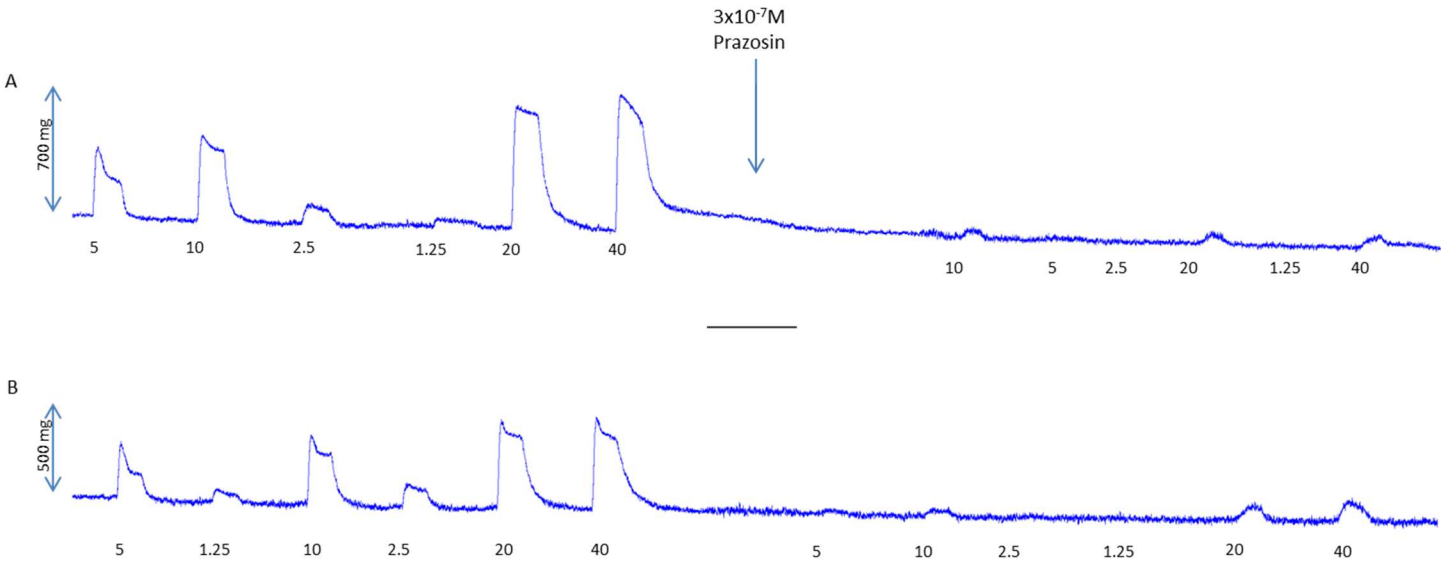

Figure AII: Representative tracing of the effect of prazosin on the field stimulation-evoked contractions at 50 V and 0.5 ms pulse width in tail arteries of CFA-treated (A) and saline-treated (B) rats at frequencies indicated.

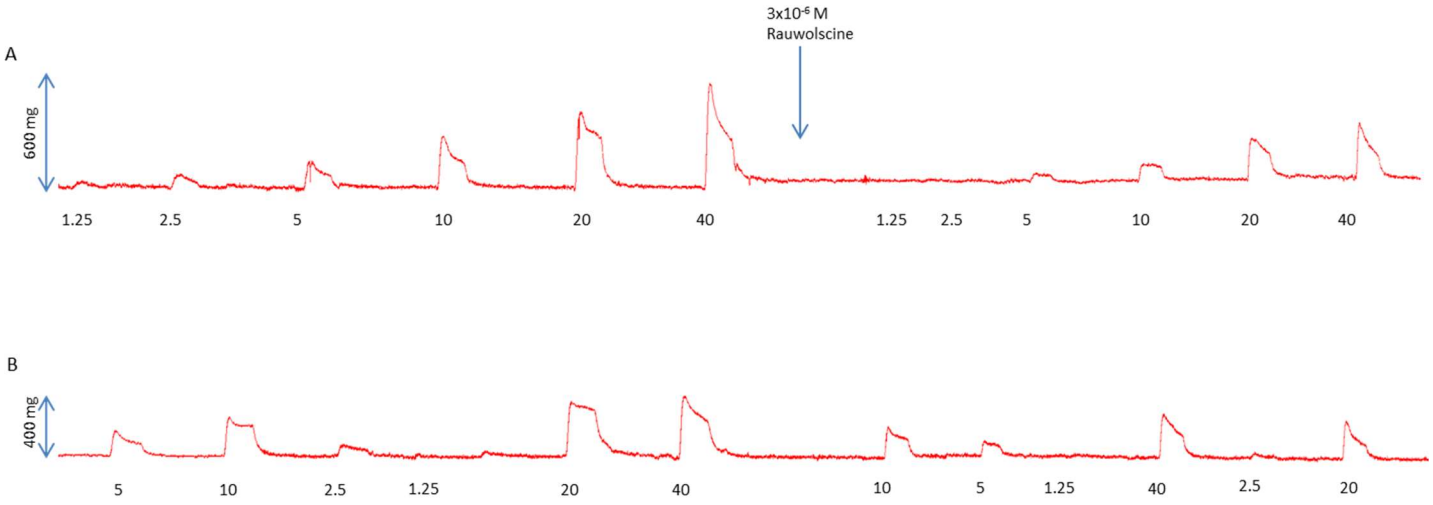

Figure AIII: Representative tracing of the effect of rauwoslcine on the field stimulation-evoked contractions in tail arteries of CFA-treated (A) and saline-treated (B) rats at frequencies indicated.